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In vitro evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts

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ABSTRACT

The cytocompatibility of novel chitosan derivative sheets and pastes were evaluated *in vitro* for possible utilization in wound dressing applications for wound healing. In this study, the cytotoxicity of oligo-chitosan (O-C), *N,O*-carboxymethyl-chitosan (NO-CMC) and *N*- carboxymethyl-chitosan (N-CMC) derivatives in sheet like and paste forms were evaluated using primary normal human dermal fibroblast cultures and hypertrophic scars; a fibrotic conditions representing a model of altered wound healing with overproduction of extracellular matrix and fibroblast hyperproliferative activity. Cytotoxicities of these chitosan derivatives were assessed using 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The results indicate that both chitosan derivative sheets and pastes have appropriate cytocompatibility and appear promising as safe biomaterials with potential wound healing applications. The SH120 (NO-CMC) derivatives sheet exhibited highest cytocompatibility property and may be regulated by MMP-13 in controlling the cell growth and its expression level.

1. Introduction

Chitosan, a de-*N*-acetylated analog of chitin, is a heteropolysaccharide consisting of linear β-1,4-linked GlcN and GlcNAc units and is widely distributed in nature as the principal component of crustaceans and insects exoskeletons as well as the cell walls of some bacteria and fungi (Jang, Kong, Jeong, Lee, & Nah, 2004). Because chitosan is relatively insoluble, its potential applications are also limited. To exploit the unique properties of these versatile polysaccharides, attempts are being made to derivatize them. Chitosan and modified derivatives display excellent biological properties including biodegradability in the human body (Sashiwa, Saimoto, Shigemasa, Ogawa, & Tokura, 1990; Shigemasa, Saito, Sashiwa, & Saimoto, 1994), as well as immunological (Mori et al., 1997; Nishimura et al., 1984), antibacterial (Tanigawa, Tanaka, Sashiwa, Saimoto, & Shigemasa, 1992; Tokura, Ueno, Miyazaki, & Nishi, 1997), and wound-healing activities (Khnor & Lim, 2003; Kweon, Song, & Park, 2003; Okamoto et al., 1993), and thus, these compounds possess unlimited application potential for use in a wide range of fields (Harish Prashanth & Tharanathan, 2007). The biodegradability, biocompatibility and non-toxicity of chitosan and its derivatives allow for widespread applications in wound healing (Muhammad, Lim, & Khor, 2001). Chitosan has been shown to posses both material and bioactive properties that may enhance wound repair (Mattioli-Belmonte et al., 1997) and the ability of chitosan and chitin to form gels, films, fibers, and sponges demonstrate their inherent versatility (Majeti & Kumar, 2000; Nakade et al., 2000; Yoshikawa, Otsuki, Midorikawa, & Terashi, 1997).

Biocompatibility has been defined as "the ability of a material to perform with an appropriate host response in a specific application" (Williams, 1987) and is one of the important prerequisites for the usefulness of biomaterials. Several procedures have been described using cell culture techniques for preliminary biocompatibility evaluation of materials intended for medical application (Pizzoferrato et al., 1994). Because much of the work describing the effects of chitosan was performed on specific cell lines or animal primary fibroblasts, fibroblasts primarily isolated from human skin cells were used in this study (Chung, Schmidt, Hamfyn, & Sagar, 1994; Mori et al., 1997; Schmidt et al., 1993). Fibroblasts

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are crucial to various organism processes that range from synthesizing ECM to mediate ECM remodeling by cytokine and metalloproteinase activity in the wound healing process (Thomas, O'Neil, Harding, & Shepperd, 1995). Fibroblast hypertrophic scars are a form of excessive dermal fibrosis and cutaneous scarring (Amadeu, Braune, & Porto, 2004) caused by deregulation of cellularity increases and decreases during the wound healing process (Sahl & Clever, 1994; Szulgit, Rudolph, & Wandel, 2002; Tanaka, Hatoko, & Tada, 2004).

The determination of cell viability is a mean of observing the *in vitro* cytotoxicity of biomaterials through detrimental intracellular effects on mitochondria and metabolic activity. The colorimetric MTT test, based on the selective ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide into purple formazan has been used to screen the *in vitro* cytotoxicity of biomaterials (Mosmann, 1983).

Currently, studies that investigate the effect of chitosan and its derivatives on primarily cultured human skin cells are limited. Furthermore, very few studies have assessed the compatibility of chitosan with human hypertrophic scars or investigated the final product of chitosan derivatives in sheet and paste forms. Thus, the purpose of this study is to evaluate the cytocompatibility of novel chitosan derivative (O-C, NO-CMC and C-MC) sheets and pastes on primary human dermal fibroblasts and hypertrophic scars.

The mechanism involved in exhibiting cytocompatibility effects by selected chitosan derivatives that exhibited highest cytocompatibility property need to be elucidated. At present, very little information is available on the mechanisms controlling matrix metalloproteinase-13 (MMP-13) expression in both normal and pathological conditions. The MMP-13 has broad substrate specificity for collagen and other ECM macromolecules and has a pivotal role in wound healing and the pathogenesis of invasive cancers and arthritis (Ala-aho & Kahari, 2005; Leeman, Curran, & Murray, 2002).

2. Materials and methods

2.1. Materials

The sterile (irradiated) sheet-like and paste-like (Fig. 1) of novel chitosan derivatives oligo-chitosan (O-C), *N*,*O*-carboxymethyl-chitosan (NO-CMC) and *N*-carboxymethyl-chitosan (N-CMC) were tested in this study. The samples were prepared and derivatized using new formulation and/or method of preparation to form sheet-like and paste-like final product (Section 2.1.1). The O-C was prepared using enzymatic reactions to achieve the desired viscosity (which is related to the molecular weight) of the oligo-chitosan solution to obtain a structurally suitable film which has not been reported before. The novelties of NO-CMC and N-CMC are the crosslinking of both polymers with PVP and PEG through gamma ray. In addition, the source of chitosan was from local shrimp exoskeleton. General information regarding sample composition is shown in Table 1. Each chitosan derivative sheet was cut into 0.5 cm × 0.5 cm square pieces.

2.1.1. Preparation of sheets and pastes

Chitosan sheet was prepared by dispersing 5 g chitosan powder in 150 ml isopropanol, while stirring with 13 ml of 20% (w/v) sodium hydroxide solution for another 1 h at room temperature. Then 6 g sodium monochloroacetate was added and heated about 1–2 h at 55 °C. It was washed with 100 ml aqueous ethanol for three times and dried in oven at 60 °C. On the other hand, chitosan paste was prepared by dispersing O-C (MW 45,660 Da), NO-CMC and N-CMC in distilled water. PVP was dissolved (Average MW 30 kDa, K 90, B.P.) into chitosan solution, then PEG was added (Average MW 200–500, Lutrol E400 BASF) at equal percentage to PVP in the range of 11%:11% to 14%:14% by weight/volume. The solution was stirred well until smooth. The pH was adjusted to about 5–6 before pouring into container. Final concentration of the water soluble chitosan/chitosan derivatives was in the range of 1–5%. The mixture was irradiated using gamma rays at 25 kGy to crosslink as well as to sterilize it in its final package.

Table 1
Properties of chitosan derivative sheets and pastes. O-C, oligo-chitosan; NO-CMC, *N*,*O*-carboxymethyl-chitosan; N-CMC, *N*-carboxymethyl-chitosan; PVP, polyvinyl pyrrolidone; PEO, polyethylene oxide; SH, sheet; P, paste.

No.	Sample code	Properties
1	SH120	NO-CMC, 3 phr PVP
2	SH121	NO-CMC, 5 phr PVP
3	SH123	O-C (3 h), PEO, PVP
4	SH124	O-C (6 h), PEO, PVP
5	P133	1% O-C, 11% PVP
6	P134	2% NO-CMC, 11% PVP
7	P136	1% NO-CMC, 11% PVP
8	P137	1% N-CMC, 11% PVP
9	P138	1% O-C, 14% PVP
10	P139	2% NO-CMC, 14% PVP
11	P141	1% NO-CMC, 14% PVP
12	P142	1% N-CMC, 14% PVP

phr, par per hundred = specific weight of material in 100 ml solution; h, hour of degradation process using chitanese enzyme.

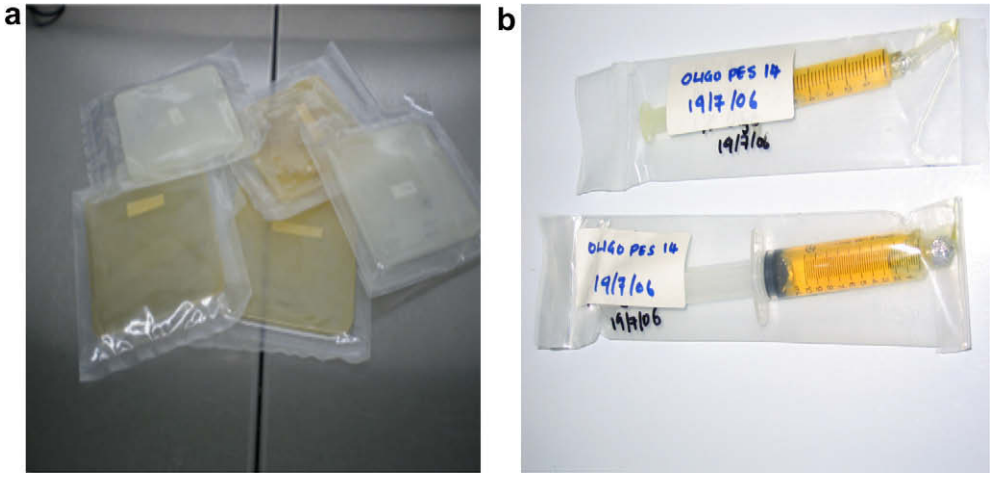

Fig. 1. Samples of chitosan derivatives (a) sheets and (b) pastes.

2.2. Primary cultures of primary human dermal fibroblasts and hypertrophic scar dermal fibroblasts

Skin material for primary culture of fibroblasts for both normal and hypertrophic scars was obtained from patients undergoing elective surgery in Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan, Malaysia. These specimens were used after patients read and signed consent forms. Sample selection was based on the inclusion and exclusion criteria and approved by the Human Ethical Committee of Universiti Sains Malaysia, USM/PPSP/Ethics Com/2003(115.4[5]).

All procedures, including reagent preparations, were performed under aseptic conditions in a Type II Biohazard Safety Cabinet. Skin samples were placed in Defined Keratinocyte Serum Free Media (DKSFM) (Gibco, BRL) supplemented with gentamicin (Gibco, BRL). Samples were sterilized by rinsing with Dulbecco's Phosphate-Buffered Saline (D-PBS) (Gibco, BRL) supplemented with gentamicin followed by 70% alcohol and again with PBS to remove excess alcohol. In order to facilitate the enzymatic digestion process, the skin samples were cut into $4 \times 4\ mm^2$ squares and transferred into DKSFM containing 2.4 U/ml of Dispase (Gibco, BRL). Samples were then incubated for 18 h at 4 °C and fibroblasts were separated from keratinocytes. Dermal layers were placed into a Petri dish containing collagenase (Gibco, BRL) (200 U/ml) solution. The petri dish was left semi-opened and incubated overnight at 37 °C in humidified atmosphere containing 5% CO_2. After incubation, fibroblasts were aspirated using pipette to dissociate the cells. The cell suspension was transferred into a 50 ml centrifuge tube followed by centrifugation at 2000 rpm for 10 min then the supernatant was removed and the cell pellet resuspended in PBS. The cell suspension was filtered through a cell strainer to remove cell debris and centrifuged at 1000 rpm for 10 min and the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL) and 100 μg/ml penicillin–streptomycin (Gibco, BRL) or complete media after the supernatant had been discarded. Cell concentration was determined using a hemocytometer and cells were seeded into culture flask at density approximately 2×10^5 viable cells/ml. Fibroblasts were maintained in complete DMEM in the 5% CO_2 incubator at 37 °C and cell morphology was observed using a light microscope.

2.3. Treatments of primary human dermal fibroblasts via direct contact

The cytotoxicity of chitosan derivative sheets and pastes was evaluated based on a procedure adapted from the International Organization for Standardization (ISO 10993-5) (1992) standard test method. Cells at the third to sixth passages were seeded into 24-well flat bottom plates at 5×10^4 viable cells/ml and incubated at 37 °C and 5% of CO_2 until the cells in each well reached 60% confluence. The medium was discarded before freshly supplemented DMEM was added to each well. Pieces of chitosan sheets were gently placed over the cultured monolayer in each well using forceps while 0.5 ml of chitosan paste was added to each well. Each chitosan derivative was assayed in triplicate in each 24-well plate. Wells without test samples served as controls. Low density polyethylene (LDPE) that does not produce a cytotoxic response was used as the negative control material. Polyvinyl chloride (PVC) was used as the positive control material. The duration of the treatments was 24, 48 and 72 h.

2.4. Cell viability assay using colorimetric tetrazolium salt (MTT)

The number of living cells was quantified with 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) assay. This quantitative assay is based on the cleavage of a yellow tetrazolium salt to insoluble purple formazan crystals by the mitochondrial dehydrogenase of viable cells. The assay was carried out according to Mosmann (1983), with some modifications. MTT (Sigma, USA) was dissolved in D-PBS and filter sterilized. At the end of each exposure period (24, 48 and 72 h) of post-treatment, pieces of chitosan sheets and pastes were removed from each well. Then, 100 μl of MTT solution was added into each well to a final concentration of 0.5 mg MTT per ml and the cultures were incubated at 37 °C, 5% of CO_2 for an additional four hours in the dark. After incubation, the culture medium in each well was removed and subsequently replaced with 1 ml of dimethylsulfoxide (DMSO) followed by agitation with a shaker to dissolve the formazan purple crystals. Later, 100 μl from each well (24-well plate) was transferred into a 96-well flat-bottom plate. The absorbance of the solution was measured at 570 nm with a reference wavelength at 690 nm using a microplate reader (Tecan, Switzerland). The relative cell growth (%) relative to control wells containing cell culture medium without test material was calculated as follows:

$$\frac{\text{Absorbance of treated cells(chitosan sheets or pastes)}}{\text{Absorbance of control}} \times 100\%$$

2.5. Western blot analysis for MMP-13

Lysate of normal and hypertrophic scar fibroblasts treated (SH120) with chitosan were subjected to SDS–PAGE analysis, followed by Western blotting assay using MMP-13 antibody (Calbiochem, Germany) at dilution 1:1000. Acrylamide gel contained proteins after electrophoresis was transferred onto polyvinylidene fluoride (PVDF) membrane immobilon P (Millipore, USA) using a semi dry electroblotting apparatus (Amersham-Pharmacia Biotech, UK) using *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) blotting buffer (10 mM CAPS, pH 11, 2.5% (v/v) methanol) for 1 h at 56 mA. Membrane was then blocked with blocking buffer [1× tris buffered saline (TBS), 0.5% (w/v) non-fat milk (Marvel), 0.1% (v/v) Tween-20] for 30 min at room temperature and washed three times (10 min each) with washing buffer [0.05% (v/v) Tween-20, 1× TBS]. After the third wash, the membranes were incubated for an hour with primary antibody followed by washing (three times, 10 min each) with washing buffer. The membranes were then incubated with the secondary antibody for one hour, followed by washing as previously described. Each membrane were then incubated with a mixture of ECL Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and then exposed to X-Omat LS Hyperfilm (Kodak, USA). The film was then developed and the produced bands were visualized under normal light.

2.6. Statistical analysis

The mean ($n = 3$) ± standard deviation (*SD*) of absorbance values measured by microplate reader was calculated. Average percentages from triplicate cell samples were analyzed using Graphpad Prism software (USA). Paired student's *t*-test was also included for statistical analysis. Differences were regarded as significant at $p < .05$.

3. Results

The cytotoxicity of chitosan derivative sheets of NO-CMC with different concentrations of polyvinyl pyrrolidone (PVP) (SH120 and SH121) at 24, 48 and 72 h are shown in Fig. 2. Both SH120 and SH121 produced significantly higher relative primary normal human dermal fibroblast cell viabilities compared to controls at 24 h ($p < .05$). Both O-C sheet derivatives (SH123 and SH124)

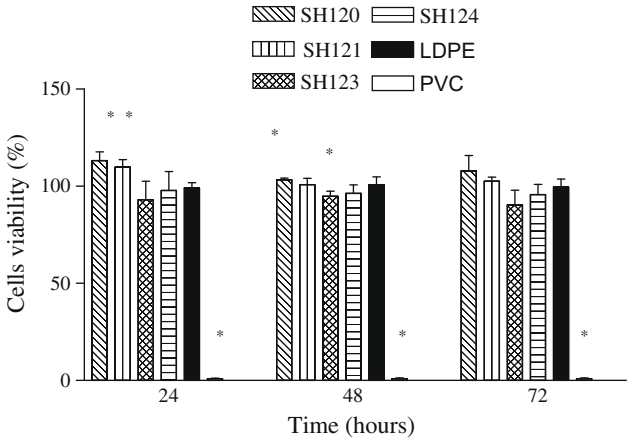

Fig. 2. Relative viability of primary normal human dermal fibroblast treated with SH120, SH121, SH123, SH124, LDPE and PVC at 24, 48 and 72 h. Data ($n = 3 \pm SD$) are presented as a percentage of control values ($^{*}p < .05$).

showed viability percentages less than 100% compared to control at 24, 48 and 72 h (Fig. 2).

The effects of other CMC derivatives in paste form were illustrated in Fig. 3. Pastes with NO-CMC, 2% (P134) and 1% (P136) concentrations with 11% PVP were evaluated. The relative viability of cells treated with P134 was 93%, 97% and 99% at 24, 48 and 72 h, respectively, while cells treated with P136 was 93%, 94% and 92%, respectively. Inhibition of cell growth by P136 was significant at all timepoints tested ($p < .05$). Normal human fibroblast cells treated with P139 and P141 showed relative cell growth less than 100% after treatment with P139 (14% PVP and 2% NO-CMC) and P141 (14% PVP and 2% NO-CMC) (Fig. 3). On the other hand, Fig. 3 showed less than 100% in cell viability upon incubation with P137 (N-CMC derivatives) compared to controls. The relative cell viability obtained from another N-CMC derivative (P142) with higher concentration of PVP (14%) was 103%, 99% and 102% respectively at 24, 48 and 72 h (Fig. 3). MTT assay did not show differences in the viability of cells treated with P142 between 24, 48 and 72 h. However, P137 significantly inhibited cell growth relative to controls at 48 and 72 h ($p < .05$).

Oligo-chitosan (O-C) derivative paste forms were also evaluated via MTT assay. As shown in Fig. 4, the cell viability compared to controls after treatment with P133 (1% oligo-chitosan and 11% PVP) was about 92%, 100% and 98% at 24, 48 and 72 h, respectively. The relative viability percentages at 24, 48 and 72 h were 93%, 102% and 97%, respectively for cells treated with P138 (1% oligo-chitosan and 14% PVP).

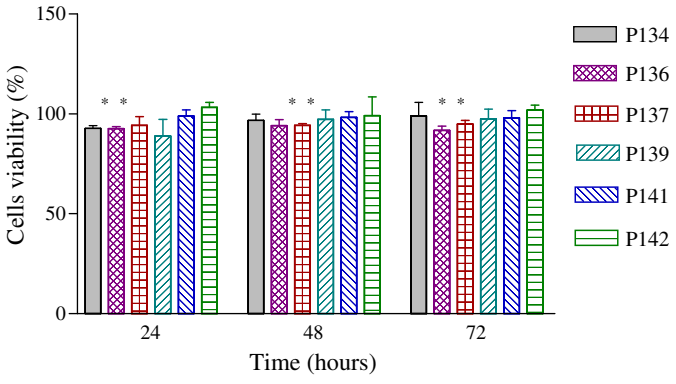

Fig. 3. Relative viability of primary normal human dermal fibroblast treated with P134, P136, P137, P139, P141 and P142 at 24, 48 and 72 h. Data ($n = 3 \pm SD$) are presented as a percentage of control values ($^{*}p < .05$).

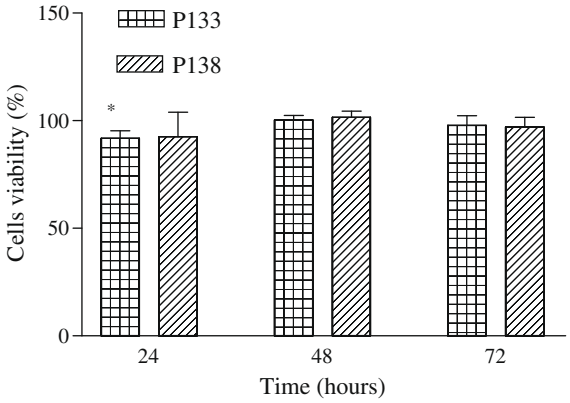

Fig. 4. Relative viability of primary normal human dermal fibroblast treated with P133 and P138 at 24, 48 and 72 h. Data ($n = 3 \pm SD$) are presented as a percentage of control values ($^{*}p < .05$).

On the other hand, when established primary human hypertrophic scar dermal fibroblast skin cells were treated with SH120 (NO-CMC derivatives sheet), the percentage of cell viability compared to control was 97%, 99% and 95% at 24, 48 and 72 h, respectively (Fig. 5). The relative viability percentage of cells treated with SH121 was 97%, 94% and 92% at 24, 48 and 72 h, respectively (Fig. 5). SH120 and SH121 significantly inhibit hypertrophic scar growth at 72 h post-treatment. For O-C derivative sheets (SH123 and SH124), growth inhibition with relative cell viability percentages of 89% and 87% at 24 and 72 h, respectively were shown. However, no significant differences relative to control were observed when hypertrophic scars were treated with SH124.

Low density polyethylene (LDPE) which served as a negative control allowed about 100% cell viability compared to controls ($p > .05$) while polyvinyl chloride (PVC) which served as positive control, produced to about 1% cell viability compared to controls ($p < .05$) at 24, 48 and 72 h (Fig. 2).

On the other hand, the MMP-13 expression of primary normal human dermal fibroblast and primary hypertrophic scar dermal fibroblast at different time points, treated with chitosan (SH120) was confirmed by immunoblotting. It demonstrated that the MMP-13 expression was detected in both samples at 24, 48 and 72 h, respectively except at 24 h for normal fibroblasts (see Fig. 6).

4. Discussion

The cytocompatibility and cell viability of chitosan derivative sheets and pastes were determined using MTT assays. This assay

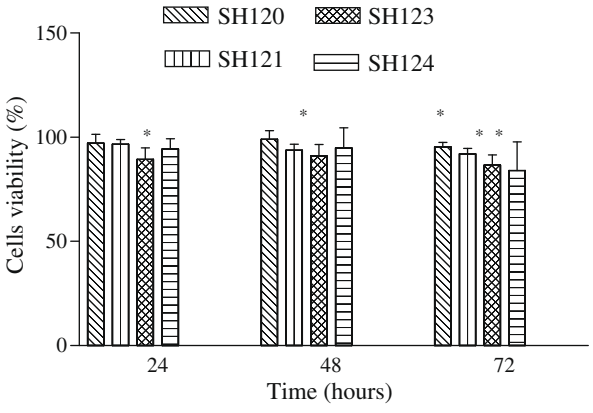

Fig. 5. Relative viability of primary human hypertrophic scar dermal fibroblast treated with SH120, SH121, SH123 and SH124 at 24, 48 and 72 h. Data ($n = 3 \pm SD$) are presented as a percentage of control values ($^{*}p < .05$).

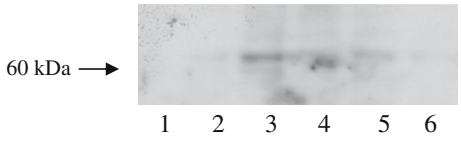

Fig. 6. MMP-13 expression of primary normal human dermal fibroblast (lanes 1–3) and hypertrophic scar dermal fibroblast (lanes 4–6) respectively at 24, 48 and 72 h treated with chitosan derivative. Dilution of MMP-13 primary antibody (1:1000).

was chosen to measure cell viability because it relies on binding to live mitochondria of living cells. MTT is specifically cleaved in living mitochondria and is chemically reduced to generate purple formazan crystals. Therefore, increased intensity of absorbance reflects increased cell numbers. The MTT assay was described as a suitable method for the detection of biomaterial cytotoxicity (Zange & Kissel, 1997) and is widely used because of its precision and reproducibility for measuring cell viability.

Both chitosan derivative sheets (SH120 and SH121) which both contained NO-CMC, but had different PVP concentrations were found to be cytocompatible. Likewise, both sheets stimulated pNHDF cell growth significantly at 24 h of treatment. The relative cell viability was higher when treated with SH120 (less PVP) versus SH121 at 24, 48 and 72 h, respectively. Enhanced growth of pNHDF could thus be attributed to the presence of PVP and could be due to PVP leaching from the sheet network, which has been shown to support cellular proliferation (Hong, Chirila, Fitton, Ziegelaar, & Constable, 1997). PVP is one of the most widely used polymers in medicine because of its solubility in water and its extremely low cytotoxicity (Lopes & Felisberti, 2003; Rosiak & Ulanski, 1999). The chitosan-PVP hydrogel was reported to be non-toxic and biocompatible with fibroblasts (NIH3T3) with the ability to modulate fibroblast growth (Risbud, Hardikar, Bhat, & Bhonde, 2000). Addition of PVP to chitosan results in hydrogels that are superior to those generated with unmodified chitosan in mechanical strength, hydrophilicity and water content (Risbud and Bhat, 2001).

In addition to sheet-based assays on CMC derivatives which showed good cytocompatibility, the cytocompatibility of CMC derivatives in paste form was also evaluated. It was observed that the relative normal fibroblast cell viability obtained from both chitosan P134 and P136 were lower than that obtained from the control. Pastes containing NO-CMC and 11% PVP indicate the cytocompatible nature of samples with growth inhibition pattern of cells viability. In addition, different concentrations of PVP do not significantly influence the viability of cell growth. When P139 and P141 pastes (2 and 1% NO-CMC, respectively) with higher concentrations of PVP (14%) were applied, the cell viability of normal fibroblasts was almost equal to that of controls. The other CMC sample, P137, which contains N-CMC with 11% PVP, showed growth inhibition pattern of relative cell viability. In contrast, N-CMC paste (P142) with a higher PVP content (14%) did not show any significant differences as compared to control. However, both appeared to be cytocompatible.

Carboxymethyl chitosan (CMC), used in preparing chitosan sheets and pastes, is a very important chitosan derivative that displays favorable water solubility and biocompatibility (Pang, Chen, Park, Cha, & Kennedy, 2007). CMC is soluble over a wide range pH and its antimicrobial activity, film-forming ability and capacity to interact with different substances can be explored in some applications where chitosan has been limitedly applied due to its restricted solubility (Liu, Guan, Yang, Li, & Yao, 2001), such as medical and pharmaceutical fields. NO-CMC is a water soluble chitosan derivative bearing carboxymethyl substituents at the amine and hydroxyl sites of glucosamine units. In addition to being soluble in water, it also has many attractive physical and biological properties, such as moisture retention, gel-forming capability, low toxicity and good biocompatibility, all of which make it a promising biomaterial. Earlier studies demonstrated that chitin and NO-CMC promote the proliferation of skin fibroblasts; additionally, chitin was proven to stimulate the migration of fibroblasts, thus accelerating wound healing (Chung et al., 1994). NO-CMC derivatives films were reported as cytocompatible to human epidermal keratinocytes cells (Lim, Halim, Lau, Ujang, & Hazri, 2007).

Neither O-C sheet (SH123 or SH124) displayed significant differences in normal fibroblast growth relative to controls at different incubation times. However, the degradation time of oligo-chitosan may influence cells viability. The longer degradation time of chitosan in SH124 demonstrated higher cell viability percentages compared to SH123 with a quicker degradation time. For both P133 and P138 O-C pastes, a similar growth pattern to controls was observed and found to be cytocompatible. Oligo-chitosan, obtained by hydrolysis or degradation of chitosan, is biodegradable, non-toxic, biocompatible and possesses versatile functional properties (Kim & Rajapakse, 2005). Similarly, Lim et al. (2007) reported that O-C derivative films were cytocompatible with keratinocytes.

NO-CMC (SH120 and SH121) derivative sheets demonstrated growth inhibition patterns relative to control for primary human hypertrophic scar dermal fibroblasts. Only O-C sheet SH123 demonstrated relative growth inhibition compared to controls. However, both NO-CMC and O-C sheets were cytocompatible. NO-CMC sheets, especially SH120, displayed cytocompatibility on both normal and hypertrophic scar fibroblasts, but inhibited the growth of hypertrophic scars at 72 h only, while stimulating the growth of normal fibroblasts at 24 and 48 h, which indicates the potential of this material for use in wound dressings. The normal fibroblast growth stimulatory effect of SH120 was only significantly shown at 24 and 48 h but not at 72 h because the total confluence was achieved in the culture well. In the case of hypertrophic scar, SH120 did not effectively inhibit the cell growth at 24 and 48 h because of the hyperproliferative activity of the cells. It was found effectively to inhibit the cell at 72 h only. It is possible that SH120 controls hypertrophic scars by inhibiting excessive cell growth while simultaneously promoting growth of normal fibroblasts cells. Reports suggest that chitosan could inhibit the growth, proliferation, biosynthesis and secretion of fibroblasts, and it may be used to treat different scars (Zhang, Xing, Sun, Song, & Ouyang, 2004). Considerable research has been focused on growth inhibition of the keloid fibroblast and growth promotion of the normal skin fibroblast during the wound healing process (Nirodi et al., 2000). Previous literature on CMC indicated that it was cytocompatible with normal skin fibroblasts and promoted their proliferation inhibiting the proliferation of keloid fibroblasts (Chen, Wang, Liu, & Park, 2002).

Polyethylene (a known biocompatible material) was used as a negative control in the cytotoxicity assay because it typically represents the extremes of non-toxicity in ISO 10993-5 standardized cytotoxicity tests ISO 10993-5 (1992). The negative control demonstrates background response of the cells and confirms cell viability is not directly affected by the mechanical stress induced by application of the test material. Negative control material also incites a reproducible non-cytotoxic response from primary normal human dermal fibroblast skin cells with a consistent viability percentage of 100% compared to control. Organo-tin PVC (a known cytotoxic material) was used as a positive control and was chosen for the cytotoxicity assay because it typically induces extreme toxicity in ISO 10993-5 standardized cytotoxicity tests ISO 10993-5 (1992). PVC prevented fibroblast cell growth, indicating the release of soluble toxic compounds thus serving its purpose in demonstrating appropriate test system response.

Our results indicate that O-C, NO-CMC and N-CMC derivatives in sheet and paste forms were cytocompatible. There are many advantages of wound dressings composed of biological materials as opposed to synthetic polymers for use in wound healing applications.

The use of chitosan and derivatives as biomaterials for wound healing has been widely reported. A very interesting property of chitosan is that it can be produced in various forms including powder, paste, film, and fiber to accommodate a variety of uses in wound healing and as wound dressing. In this study, sheet and paste forms of different chitosan derivatives were tested *in vitro*, and the observations regarding their effects on cells in culture may provide a useful screen for potential *in vivo* activity as wound healing agents. The *in vitro* results obtained herein correspond to published data on tissue compatibility in animal models (Zange & Kissel, 1997). It has been shown that some derivatives of chitosan are capable of accelerating wound healing (Biagini, Bertani, & Muzzarelli, 1991; Muzzarelli et al., 1990; Muzzarelli, Weckx, & Biachiega, 1991). In fact, the Hypecan cap, an occlusive biological fingertip dressing based on chitin, effectively restored functional and cosmetic properties of skin in a relatively short time span and resulted in less pain and discomfort compared to conventional dressings (Halim, Stone, & Devaraj, 1998).

Fibroblasts were chosen in this study because they are considered to be critical in the wound healing process. It is well accepted that fibroblasts play critical roles in various processes ranging from ECM synthesis to mediating ECM remodeling by cytokine and metalloproteinase activity (Thomas, O'Neil, Harding, & Shepperd, 1995). Dermal fibroblasts have numerous functions, including proliferation and migration in response to chemotactic, mitogenic and modulatory cytokines, as well as autocrine and paracrine interactions (Clark, 1996). A number of researchers (Chen et al., 2002; Mori et al., 1997) have adopted fibroblast cell growth as a criterion for evaluating the wound healing ability of a polymeric material. Previously, very few studies focused on human hypertrophic scar dermal fibroblasts. The early migration and proliferation of fibroblasts in the wound area is implicated in wound scarring (Risbud et al., 2000) and a comparison of healing trends between dressing materials from chitosan and both synthetic and naturally derived commercial materials has shown the advantages of chitosan dressing as assayed by healing rate, histological healing and scar colour (Stone, Wright, Clarke, Powell, & Devaraj, 2000). Chitosan-based wound dressings have been shown to reduce scar tissue (fibroplasias) by inhibiting the formation of fibrin in wounds, have also been shown to be hemostatic, forming a protective film coating on the wound (Lloyd, Kennedy, Methacanon, Paterson, & Knill, 1998).

Primarily fibroblasts isolated from human skin cells were used in this study since much of the previous work describing the effects of chitosan was performed on specific cell lines, including L929 mouse fibroblasts (Mori et al., 1997; Schmidt et al., 1993), F1000 embryonic skin and muscle fibroblasts (Chung et al., 1994), or fibroblasts from animals such as rats or mice (Mori et al., 1997). Advantage of studying fibroblasts obtained from primary culture is that they contain normal diploid complement of chromosomes, and therefore, in this respect, mimic fibroblasts *in vivo* (Rittie & Fisher, 2005). In contrast, fibroblast cell lines typically have numerous chromosomal aberrations and mutations, which are associated with their ability for unlimited growth (Rittie & Fisher, 2005). Primary cultures of hypertrophic scar, a fibrotic condition representing a model of altered wound healing with overproduction of extracellular matrix and fibroblast hyperproliferative activity, were used to compare with normal fibroblasts which were also isolated primarily. It has a potential to be used in research and treatment since very few study focus on pathogenesis of hypertrophic scar. Moreover, none commercial hypertrophic scar cell line is available.

In vitro, the effects of chitin and chitosan on fibroblasts have been studied and similar to *in vivo* studies, both stimulatory and inhibitory actions have been reported. Chitosan can inhibit fibroblast proliferation (Mori et al., 1997; Schmidt et al., 1993; Xia, Hou, & Wang, 2007) as demonstrated using high chitosan concentrations in cultures to demonstrate a significant reduction in the rate of L929 fibroblast proliferation (Mori et al., 1997). Chitosan showed stimulatory effects on fibroblasts, but inhibited human keratinocyte proliferation (Howling et al., 2001). Furthermore, Chung et al. (1994) reported a stimulatory effect of chitosan. These seemingly contradictory data may partially result from the different chemical compositions and physical forms of the biopolymer samples investigated, making it difficult to clarify the relationship between chitosan structure and its effects on fibroblast behavior (Howling et al., 2001). These results may also be explained by differences in the cell populations analyzed and by variation in the properties of chitosan materials. As shown in this study, different patterns of cell growth were demonstrated in response to chitosan derivative sheet and paste treatments, but all samples were found to be cytocompatible.

Mechanisms and functions underlying NO-CMC remain unknown and require further research. We previously reported that CTCF, YB-1, P53 and c-Myc may play role in controlling cells growth in response to SH120 (NO-CMC) sheets (Mohammad Syaiful Bahari et al., 2008). In this study, the MMP-13 was detected in chitosan treated primary human dermal fibroblasts and in pathologic condition of human skin, the hypertrophic scar cells. Fibroblasts synthesized collagen and increased after a wound to remodel the injured area (Parsonage et al., 2005; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002) and at the same time, the cells also produce MMP or collagenases (ECM-degrading protease) which function to maintain an ECM homeostasis by regulation ECM turnover (Chang et al., 2002; Simian et al., 2001). The expression of MMP-13 protein of chitosan treated fibroblasts, suggesting a significant role for this protein in ECM homeostasis and wound healing activity. MMP-13 was not detected when treated with chitosan SH 120 at 24 h but found to be expressed later. Chitosan inhibited MMP-13 expression but at the same time did not inhibited normal fibroblast cells. In this context, chitosan when treated in normal cells (<48 h) inhibited MMP-13 expression. It is suggested that the chitosan may be useful inhibiting the MMP-13 or collagenase production which affect degrading activity of ECM but without inhibit fibroblasts proliferation. Chitosan treatment may only delay MMP-13 expression as it found to be expressed later (after 24 h). MMP-13 expression was also found to be expressed in hypertrophic scar samples treated with chitosan. The expression may degrade the collagen overexpressed in wound healing process of hypertrophic scars by inhibiting hypertrophic scar cell viability. It may be useful to be used in controlling overproduction of collagen in hypertrophic scars. The MMP-13 expression showed different activity in different cell type after chitosan treatment. However, no toxicity was associated with expression of MMP-13 as judged by MTT assay for both normal and hypertrophic scar cells.

5. Conclusion

The properties of O-C, NO-CMC and N-CMC in forming sheets or pastes may lead to the preparation of new biomaterials that are expected to display the following advantages: acceptable mechanical strength, excellent biocompatibility and reduced fluid and heat loss from wounds. Both materials show promising cytocompatibility and cell viability for both hypertrophic scars and normal fibroblasts, and must be further evaluated regarding the mechanisms involved. MMP-13 may also play an important role in controlling the cell growth and its expression level may be regulated by chitosan NO-CMC (SH120). Further study would also allow for the examination of the possibility that these materials could be used to develop improved wound management products.

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References

Ala-aho, R., & Kahari, V. M. (2005). Collagenases in cancer. *Biochimie, 87*, 273–286.

Amadeu, T. P., Braune, A. S., & Porto, L. C. (2004). Fibrillin-1 and elastin are differentially expressed in hypertrophic scars and keloids. *Wound Repair and Regeneration, 12*, 169–174.

Biagini, G., Bertani, A., & Muzzarelli, R. (1991). Wound management with *N*-carboxybutyl chitosan. *Biomaterials, 12*, 281–286.

Biological evaluation for medical devices, part 5, Tests for cytotoxicity: *In vitro* methods, ISO 10 993-5, EN 30 993-5 (1992).

Chang, H. Y., Chi, J. T., Dudoit, S., Bondre, C., Van De Rijn, M., & Botstein, D. (2002). Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proceedings of National Academic Sciences United States of America, 99*, 12877–12882.

Chen, X. G., Wang, Z., Liu, W. S., & Park, H. J. (2002). The effect of carboxymethyl-chitosan on proliferation tk;1and collagen secretion of normal and keloid skin fibroblasts. *Biomaterials, 23*, 4609.

Chung, L. Y., Schmidt, R. J., Hamfyn, P. F., & Sagar, B. F. (1994). Biocompatibility of potential wound management products: Fungal mycelia as a source of chitin/chitosan and their effect on the proliferation of human F1000 fibroblasts in culture. *Journal of Biomedical Material Research, 28*, 463–469.

Clark, R. A. F. (1996). Wound repair: Overview and general considerations. In R. A. F. Clark (Ed.), *The molecular and cellular biology of wound repair* (pp. 3–35). New York, NY: Plenum Press.

Halim, A. S., Stone, C. A., & Devaraj, V. S. (1998). The Hypecan cap: A biological fingertip dressing. *Injury, 29*(4), 261–263.

Harish Prashanth, K. V., & Tharanathan, R. N. (2007). Chitin/chitosan: Modifications and their unlimited application potential – An overview. *Trends in Food Science & Technology, 18*(3), 117–131.

Hong, Y., Chirila, T. V., Fitton, J. H., Ziegelaar, B. W., & Constable, I. J. (1997). Effect of crosslinked poly(1-vinyl-2-pyrrolidinone) gels on cell growth in static cell cultures. *Bio-Medical Materials and Engineering, 7*(1), 35–47.

Howling, G. I., Dettmar, P. W., Goddard, P. A., Hampson, F. C., Dornish, M., & Wood, E. J. (2001). The effect of chitin and chitosan on the proliferation of human skin fibroblasts and keratinocytes in vitro. *Biomaterials, 22*(22), 2959–2966.

Jang, M. K., Kong, B. G., Jeong, Y. I., Lee, C. H., & Nah, J. W. (2004). Physicochemical characterization of α-chitin, β-chitin and γ-chitin separated from natural resources. *Journal of Polymer Science. Part A: Polymer Chemistry, 42*, 3423–3432.

Khnor, E., & Lim, L. (2003). Implantated applications of chitin and chitosan. *Biomaterials, 24*, 2339–2349.

Kim, S. K., & Rajapakse, N. (2005). Enzymatic production and biological activities of chitosan oligosaccharides (COS): A review. *Carbohydrate Polymers, 62*, 357–368.

Kweon, D. K., Song, S. B., & Park, Y. Y. (2003). Preparation of water-soluble chitosan/heparin complex and its application as wound healing accelerator. *Biomaterials, 24*, 1595–1601.

Leeman, M. F., Curran, S., & Murray, G. I. (2002). The structure, regulation, and function of human matrix metalloproteinase-13. *Critical Review of Biochemistry and Molecular Biology, 37*, 149–166.

Lim, C. K., Halim, A. S., Lau, H. Y., Ujang, Z., & Hazri, A. (2007). *In vitro* cytotoxicology model of oligo-chitosan and *n,o*-carboxymethyl chitosan using primary normal human epidermal keratinocyte cultures. *Journal of Applied Biomaterials, 5*, 82–87.

Liu, X. F., Guan, Y. L., Yang, D. Z., Li, Z., & Yao, K. D. (2001). Antibacterial action of chitosan and carboxymethyled chitosan. *Journal of Applied Polymer Science, 79*, 1324–1335.

Lloyd, L. L., Kennedy, J. F., Methacanon, P., Paterson, M., & Knill, C. J. (1998). Carbohydrate polymers as wound management aids. *Carbohydrate Polymers, 37*, 315–322.

Lopes, C. M. A., & Felisberti, M. I. (2003). Mechanical behaviour and biocompatibility of poly(1-vinyl-2-pyrrolidinone)–gelatin IPN hydrogels. *Biomaterials, 24*(7), 1279–1284.

Majeti, N. V., & Kumar, R. (2000). A review of chitin and chitosan applications. *Reactive and Functional Polymer, 46*, 1–27.

Mattioli-Belmonte, M., Mengucci, P., Specchia, N., Gobbi, G., Dubini, S., Simonelli, L., et al. (1997). An experimental study in X-ray spectroscopy of the zirconium (Ca-PSZ) – Bone interface. Microanalytic evaluation of the osteogenetic response. *Journal of Material Science, 8*(2), 85–90.

Mohammad Syaiful Bahari, A. R., Halim, A. S., Kamaruddin, H., Hazri, A. R., Lim, C. K., & Shaharum, S. (2008). CTCF, YB-1, C-MYC and P53 expressions of primary human hypertrophic scar and normal fibroblasts skin cells in response to novel chitosan derivatives sheet. *Chinese Journal of Biotechnology, 24*(12), 2137–2139.

Mori, T., Okumura, M., Matsuura, M., Ueno, K., Tokura, S., Okamoto, Y., et al. (1997). Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts *in vitro*. *Biomaterials, 18*, 947–951.

Mosmann, T. (1983). Rapid colorimetric assays for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55–63.

Muhammad, Y. N. L. B., Lim, L. Y., & Khor, E. (2001). Preparation and characterization of chitin beads as a wound dressing precursor. *Journal of Biomedical Material Research, 54*, 59–68.

Muzzarelli, R., Tarsi, R., Filippini, O., Giovanetti, E., Biagini, G., & Varaldo, P. E. (1990). Antimicrobial properties of *N*-carboxybutyl chitosan. *Antimicrobial Agents and Chemotherapy, 34*(10), 2019–2023.

Muzzarelli, R. A. A., Weckx, M., & Biachiega, V. (1991). N-carboxybutyl chitosan as a wound dressing and a cosmetic ingredient. *Chimicaoggi, 4*, 33–37.

Nakade, T., Yokota, H., Taniyama, H., Hori, Y., Agata, N., Ikeda, T., et al. (2000). Matrix metalloproteinase (MMP) 9 induced in skin and subcutaneous tissue by implanted chitin in rats. *Carbohydrate Polymers, 41*, 327–329.

Nirodi, C. S., Devalaraja, R., Nanney, L. B., Arrindell, S., Russell, S., Trupin, J., et al. (2000). Chemokine and chemokine receptor expression in keloid and normal fibroblasts. *Wound Repair Regeneration: Official Public Wound Healing Society European Tissue Repair Society, 8*, 371–382.

Nishimura, K., Nishimura, S., Nishi, N., Saiki, I., Tokura, S., & Azuma, I. (1984). Immunological activity of chitin and its derivatives. *Vaccine, 2*, 93–99.

Okamoto, Y., Minami, S., Matsuhashi, A., Sashiwa, H., Saimoto, H., Shigemasa, Y., et al. (1993). Polymeric *N*-acetyl-D-glucosamine (chitin) induces histionic activation in dogs. *The Journal of Veterinary Medical Science, 55*, 739–742.

Pang, H. T., Chen, X. G., Park, H. J., Cha, D. S., & Kennedy, J. F. (2007). Preparation and rheological properties of deoxycholate-chitosan and carboxymethyl-chitosan in aqueous systems. *Carbohydrate Polymers, 69*, 419–425.

Parsonage, G., Filer, A. D., Haworth, O., Nash, G. B., Rainger, G. E., Salmon, M., et al. (2005). A stromal address code defined by fibroblasts. *Trends in Immunology, 26*, 150–156.

Pizzoferrato, A., Ciapetti, G., Stea, S., Cenni, E., Arciola, C. R., Granchi, D., et al. (1994). Cell culture methods for testing biocompatibility. *Clinical Materials, 15*, 173–190.

Risbud, M. V., & Bhat, S. V. (2001). Properties of polyvinyl pyrrolidone/β-chitosan hydrogel membranes and their biocompatibility evaluation by haemorheological method. *Journal of Materials Science: Materials in Medicine, 12*, 75–79.

Risbud, M. V., Hardikar, A. A., Bhat, S. V., & Bhonde, R. R. (2000). PH-sensitive freeze-dried chitosan–polyvinyl pyrrolidone hydrogels as controlled release system for antibiotic delivery. *Journal of Controlled Release, 68*(1), 23–30.

Rittie, L., & Fisher, G. J. (2005). Isolation and culture of skin fibroblasts. In: J. Varga, D. A. Brenner, & S. H. Phan (Eds.), *Method in molecular medicine, fibrosis research: Methods and protocols* (pp. 83–98). New York: Humana Press Inc.

Rosiak, J. M., & Ulanski, P. (1999). Synthesis of hydrogels by irradiation of polymers in aqueous solution. *Radiation Physics Chemistry, 55*, 139.

Sahl, W. J., & Clever, H. (1994). Cutaneous scars: Part I. *International Journal of Dermatology, 33*, 681–691.

Sashiwa, H., Saimoto, H., Shigemasa, Y., Ogawa, R., & Tokura, S. (1990). Lysozyme susceptibility of partially deacetylated chitin. *International Journal of Biological Macromolecules, 12*, 295–296.

Schmidt, D., Messer, G., Franz, S., Walchner, M., Schuppe, H. C., Weiss, E. H., et al. (1993). Association of cutaneous and systemic forms of lupus erythematosus to genetic variations of the genes of tumor necrosis factor-α and lymphotoxin. *Journal of Dermatological Science, 6*(1), 2.

Shigemasa, Y., Saito, K., Sashiwa, H., & Saimoto, H. (1994). Enzymatic degradation of chitins and partially deacetylated chitins. *International Journal of Biological Macromolecules, 16*, 43–49.

Simian, M., Hirai, Y., Navre, M., Werb, Z., Lochter, A., & Bissell, M. J. (2001). The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development, 128*, 3117–3131.

Stone, C. A., Wright, H., Clarke, T., Powell, R., & Devaraj, V. S. (2000). Healing at skin graft donor sites dressed with chitosan. *British Journal of Plastic Surgery, 53*(7), 601–606.

Szulgit, G., Rudolph, R., & Wandel, A. (2002). Alterations in fibroblast [alpha]1[beta]1 integrin collagen receptor expression in keloids and hypertrophic scars. *Journal of Investigative Dermatology, 118*, 409–415.

Tanaka, A., Hatoko, M., & Tada, H. (2004). Expression of p53 family in scars. *Journal of Dermatological Science, 34*, 17–24.

Tanigawa, T., Tanaka, Y., Sashiwa, H., Saimoto, H., & Shigemasa, Y. (1992). Various biological effects of chitin derivatives. In C. J. Brine, P. A. Sandford, & J. P. Zikakis (Eds.), *Advances in chitin and chitosan* (pp. 206–215). Elsevier.

Thomas, D. W., O'Neil, I. D., Harding, K. G., & Shepperd, J. P. (1995). Cutaneous wound healing. *Journal of Oral and Maxillofacial Surgery, 53*, 442–447.

Tokura, S., Ueno, K., Miyazaki, S., & Nishi, N. (1997). Molecular weight dependent antimicrobial activity by chitosan. *Macromolecular Symposia, 120*, 1–9.

Tomasek, J. J., Gabbiani, G., Hinz, B., Chaponnier, C., & Brown, R. A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nature Review of Molecular and Cellular Biology, 3*, 349–363.

Williams, D. F. (Ed.). (1987). *Definitions in biomaterials*. Amsterdam: Elsevier.

Xia, P., Hou, C., & Wang, W. (2007). Inhibitive effects of chitosan on proliferation of fibroblasts *in vitro*. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi, 21*(8), 833–836.

Yoshikawa, M., Otsuki, T., Midorikawa, T., & Terashi, T. (1997). Process for producing articles of regenerated chitin-chitosan containing material and the resulting articles. *European Patent No. 794223*.

Zange, R., & Kissel, T. (1997). Comparative in vitro biocompatibility testing of polycyanoacrylates and poly(D,L-lactide-co-glycolide) using different mouse fibroblast (L929) biocompatibility test models. *European Journal of Pharmaceutics and Biopharmaceutics, 44*, 149–157.

Zhang, J. D., Xing, X., Sun, M. Q., Song, J. X., & Ouyang, T. X. (2004). The effects of chitosan on different-derived fibroblasts. *Zhonghua Zheng Xing Wai Ke Za Zhi, 20*(1), 53–56.