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THE EFFECT OF CHITOSAN DERIVATIVES FILM ON THE PROLIFERATION OF HUMAN SKIN FIBROBLAST: *AN-IN VITRO STUDY*

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Abstract: Wound healing is a natural restorative response to tissue injury. Nowadays, wound healing study has become an active area for many researchers and the development of biomaterials as wound management product has been increased. Biocompatibility is one of the most important criteria in biomaterials selection. The objective of this study is to examine the cytotoxicity effect of newly developed chitosan derivatives film on fibroblasts growth and its proliferation*in-vitro*. The effect of oligochitosan on the secretion of interleukin 8 (IL-8) and transforming growth factor beta (TGF-β) also is being evaluated. The cytotoxicity of these chitosan products were compared by measuring cell survival with the tetrazolium salt reduction (MTT) assay which measured the mitochondrial activity of active living cells. The determination of fibroblasts proliferation was done by using CellTiter 96® Aqueous Non- Radioactive Cell Proliferation Assay. The results demonstrated that oligochitosan film was non-toxic. Low molecular weight chitosan derivatives have the ability to induce the proliferation of fibroblasts and stimulation of proliferation increased with time exposure. However, no fibroblasts proliferation detected in the presence of high molecular weight chitosan derivatives during the 5 days of incubation. The secretion of IL-8 by fibroblasts was also detected at low concentration of chitosan derivatives. However, none of TGF-β was detected. This study clearly demonstrated the growth modulating effects of chitosan on fibroblasts *in-vitro*. Thus, further study should be developed in order to clarify the effect of biomedical grade chitosan for wound management applications.

KEYWORDS: Chitosan derivatives, wound healing, cytocompatibility, cell proliferation.

Introduction

Chitin and chitosan are natural biomaterials which are found ubiquitously in exoskeleton of crustaceans and also in the cell walls of fungi and insects (Prasitsilp *et al.,* 2000; Keisuke, 1998). This natural polysaccharide has been widely used in a wide variety of biomedical applications, medicine, agriculture, food and non-food industries (Rinaudo, 2006). The chitin and chitosan application versatility is due to the diverse biological activity, excellent biocompatibility and complete biodegradability in combination with low toxicity (Majeti and Kumar, 2000).

Recently, increased attention has been given to the development of new dressing to promote healing in human. Although traditional wound dressings offer dry wound healing conditions, the use of occlusive dressings, hydrocolloid and hydrogel dressings which provide moist healing conditions has increased significantly (Draye *et al*., 1998). The important criteria in the development of new dressing is the introduction of medicated wound dressing capable of delivery active substance into the wound sites, biocompatible and able to promote the production of cytokine and growth factor which are involved in healing process (Rochrich *et al.,* 1999; Romo *et al.,* 2005).

Chitin and chitosan possess several prominent features which made them important biomaterials for wound management application (Yusof *et al.,* 2003). Studies have shown that it could accelerate the tensile strength of wound by spreading the fibroblastic synthesis of collagen in the initial phase of healing, promote normal tissue regeneration and homeostasis. In addition, chitin and its derivatives also have been reported to accelerate wound healing by enhancing the function of inflammatory cells and repairing cells (Shi *et al.,* 2006). The biodegradable chitin and its derivatives also have bacteriostatic and fungistatic activities (Tomihata and Ikada, 1997).

Biocompatibility can be defined as the ability of medical devices to perform their intended function with an appropriate host response in a specific application during the whole life cycle of the device (Shayne, 2002). The medical device or their component materials may have surface characteristics that produced undesirable effect when used clinically. Therefore, an evaluation of biocompatibility is one part of the overall safety assessment of a device (International Organization for Standardization, 1992). This present study examines the cytotoxicity effect of oligo chitosan on primary fibroblast culture. Cell proliferation and the production of cytokine and growth factor (interleukin 8 and TGF-β) are also being evaluated to clarify the effect of oligo chitosan in healing process.

Materials and Methods

Biomaterial Samples

Chitosan derivatives films were prepared and supplied by Standard and Industrial Research Institute of Malaysia (SIRIM Bhd) and sterilized by ethylene oxide. Generally, oligo chitosan were prepared by an enzyme hydrolysis reaction. Pure chitosanase from *Streptomyces species* (Sigma Aldrich) was used as hydrolytic enzyme and reaction was run at 50°C. The enzymatic hydrolysis was performed in 10 liters of 3% acidic chitosan solution. Hydrolysed sample was taken at required time and inactivated at 100°C. Viscosity was measured upon cooling using Brookfield Viscometer with spindle 29 at 100 rpm. Oligo chitosan were freeze dried and refrigerated to prevent further hydrolysis. Samples with concentration of 1% to 5% solution in water were prepared for *in-vitro* analysis. Three types of each oligo chitosan were further developed based on their molecular weight named as O-C 1% (1h, 3h, and 6h) and O-C 5% (1h, 3h, and 6h). The molecular weight (MW) for each chitosan derivatives 1h, 3h and 6h are 309980, 69702 and 45660 respectively. Oligochitosan films were prepared by casting solution onto glass plate and evaporating the water in an incubator. In the preparation of modified edible films, the different composition plasticizer was added into the solution. Each of these solutions was stirred for 5 to 10 minutes, pours onto glass plate and dried in an incubator at 40^{o}C.

Primary Fibroblast Culture

Fibroblasts were cultured from healthy donor after informed consent following elective surgery procedures at Hospital Universiti Sains Malaysia (HUSM). The protocol of procedure for skin samples used for cultured was approved by the Medical Research and Ethical Committee of the Universiti Sains Malaysia (USM). Cultures were established by single-cell suspension technique following enzymatic digestion of skin samples. In general, skin tissue fragments were incubated with dispase (Gibco-invitrogen) for 12 to 18 hours and subsequently underwent treatment with *Clostridium Histolyticum* Collagenase A. This was left overnight to liberate the fibroblasts and incubated with dulbecco's modified eagle medium (DMEM) (Gibco-invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) (Gibco-invitrogen) at 37 ^{o}C in 5% CO_2. The medium was changed weekly until confluent cell monolayer was formed. After observation of the fibroblasts outgrowth, subculture was performed by removing the dermis remnants using phosphate buffered saline (PBS) solution (Gibco-invitrogen) and trypsinizing the cells. Trypsin (Gibco-invitrogen) was neutralized with DMEM complete medium and seeded at 3 x 10^6 cells per 75 cm^2 culture flask for further expansion.

Cytotoxicity Assay

The direct contact cell culture evaluation of materials was employed to assess the cytotoxicity of biomaterials (International Organization for Standardization, ISO 10993-5, 1992). Fibroblasts (6 x 10^4 cells/ml) in DMEM supplemented with 10% FBS were seeded in each well of 24-well culture plates (Nunc, Denmark). Plates were incubated overnight at 37^0C in humidified 5% CO_2 atmosphere until a monolayer was formed. The culture medium was aspirated and oligo chitosan pieces (0.5 cm^2) were placed carefully on top of the cell layer in a minimum culture medium. Cultures underwent 24, 48 and 72 hours treatment with test materials. Morphology of cells was assessed using inverted microscope and quantitative evaluation of cytotoxicity was done using tetrazolium salt (MTT) (Sigma-Aldrich) reduction assay. After the desired time exposure, 10 µl of MTT solution (5 mg/ml in PBS) was added into each well. After incubation for 4 hours at 37 ^{0}C in the dark, medium and MTT were removed from each well. Purple MTT-formazan crystal form by this reaction was dissolved by adding 100 µl of dimethylsulfoxide (DMSO) (Sigma-Aldrich). Absorbance was read at 570nm. The graph percentage of viable cells against test material concentration was plotted and calculated as follows:

$$\text{Percentage of viable cells} = \frac{(OD_{\text{sample}} - OD_{\text{medium control}})}{(OD_{\text{value}} - OD_{\text{medium control}})} \times 100\%$$

Where, OD_{sample} = Optical density of treatment samples.
OD_{value} = Optical density of control.
$OD_{medium\ control}$ = Optical density of background control.

Cell Proliferation Assay

The proliferation of fibroblasts treated with oligochitosan film was done by using celltiter 96® aqueous non-radioactive cell proliferation assay (MTS) (Promega Corporation, Fitchburg Winconsin) as manufactured instructions. A confluent monolayer of fibroblasts was trypsinized and the number of cells was adjusted at 4 × 10^4 cells in 1ml DMEM supplemented medium with 10% FBS and 1% antibiotics. The cells were then seeded into 96-well culture plates and incubated overnight in CO_2 incubator until they reached 70% to 80% confluent. On the next day, medium was discarded and replaced with 100µl of fresh DMEM medium supplemented with various concentrations of test materials extract. Incubation of cells with test materials was done for 24, 48, 72, 120 and 144 hours of exposure. Then, 20 µl of the combined MTS/PMS solution was pipetted into each well of the 96-well plates containing 100 µl of cells in culture medium. Culture plate was incubated for 1 to 4 hours. At this time, MTS is reduced into soluble formazan product by dehydrogenase enzyme activity which was produced by metabolically active cells. The absorbance was measured at 490 nm. Since the production of formazan is proportional to the number of living cells, the intensity of the produced color is a good indication of the viable cells in proliferation.

The Effect of Chitosan Derivatives Product on Interleukin 8 (IL-8) and Transforming Growth Factor ß (TGF-ß) Productions

The production of IL-8 (Bender MedSystems GmbH, California) and TGF-β (Bender MedSystems GmbH, California) by fibroblasts was done by using enzyme linked immunosorbent assay (ELISA) as manufactured instructions. Human dermal fibroblast was seeded (6 × 10^4 cells/ml) in 96-well tissue culture plates in DMEM media with 5% FBS respectively. After growing at confluence, the culture medium was discarded and replaced with the same volume of fresh medium supplemented with or without oligochitosan. After 24 hours, the supernatant was harvested and used for assays of IL-8 and TGF-bproductions. All samples, standard, blank and control samples were assayed in duplicate. Each standard and blank well were added with 100 µl of distilled water and wells designated for samples were added with 50 µl of distilled water plus 50 µl of sample and incubated for 2 hours. Then, 100 µl of tetramethylbenzidine (TMB) substrate solution was pipetted and incubated for 10 minutes. When the highest standard has developed a dark blue color, 100 µl of stop solution was added to stop the enzyme reaction. Absorbance was read on ELISA reader using 450 nm as primary wavelength and 620 nm reference wavelengths. The calculation of the result was

done by using the average of absorbance values for each set of duplicate standard and samples. The standard curved was created by plotting the mean absorbance for each standard concentration against the IL-8 and TGF-β concentration. To determine the concentration of IL-8 and TGF-β for each sample, the concentration read from the standard curve was multiplied by the dilution factor (×2) for IL-8 and dilution factor (×12) for TGF-β.

Statistical Analysis

For each microplate, reading values calculated in the exposed cells were converted into percentage, with the negative control values considered to be 100%. The data was reported as the mean ± standard deviation (SD) and analyzed with non-parametric Wilcoxon signed ranks test as significant different at $P<0.05$.

Results

Chitosan derivatives tested were found to be non-toxic to human skin fibroblasts. The results showed that all chitosan derivatives induced minimal cytotoxicity effect to fibroblasts *in-vitro* as they represent more than 80% of cells viability. Figure 1 depicts the cytotoxicity results by direct contact test for 24, 48 and 72 hours of exposure to test materials. The results demonstrated that, O-C 1% derivatives exhibit high percentage of cell viability compared to O-C 5% in terms of their cytotoxicity. However all of oligochitosan maintained high percentage of viable cells which exhibit more than 80% of viability at all time of exposures. In addition, increased in time exposure to materials extract also did not affect the percentage of viable cells.

Two derivatives of each oligo chitosan [O-C 1% (3h and 6h) and O-C 5% (3h and 6h] were selected for the proliferation study. The

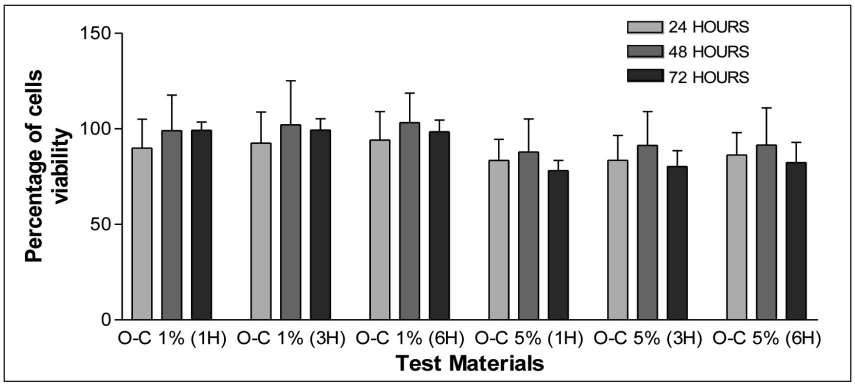

Figure 1: Quantitative Cytotoxicity Test: Fibroblasts Survivals in the Presence of Test Materials (direct contact) for 24, 48 and 72 Hours Exposure.

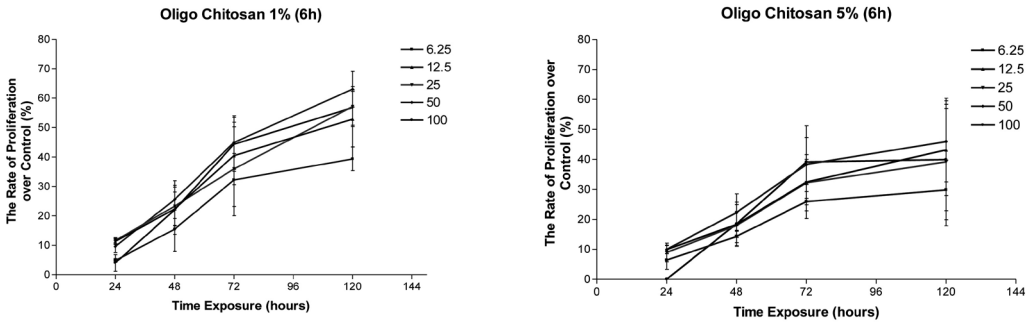

Figure 2: The Effect of Chitosan Derivatives Film on Fibroblasts Proliferation *in-vitro*. Human Dermal Fibroblasts were Treated with Various Concentrations of Samples for 5 Days.

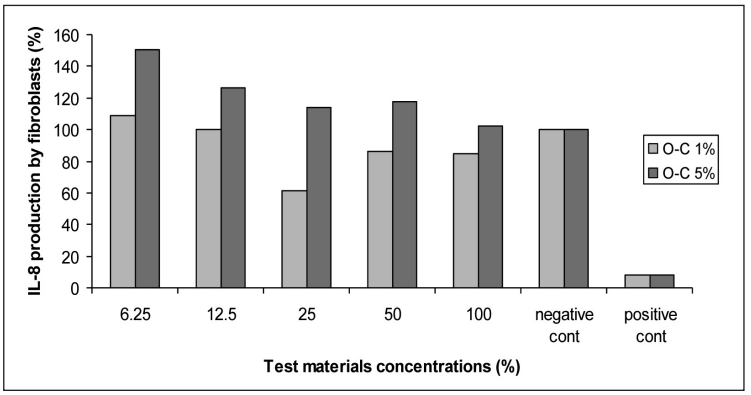

Figure 3: Percentage of IL-8 Production by Fibroblasts over Negative Control after Treated with Various Concentrations of O-C 1% and 5%.

effect of chitosan derivatives film on fibroblasts proliferation was investigated over a range of concentration (6.25% to 100% of oligochitosan extract). The results revealed that, both O-C 1% and O-C 5% (3h) did not stimulate the proliferation of fibroblasts. In addition, some of them (100% concentration) showed the reduction of viability, indicating the decrease in viable cells after 5 days of incubation.

O-C 1% and 5% (6h) showed the strongest stimulatory effects on cell proliferation at all test material concentrations tested (Figure 2). The rate of proliferation also increased with time. The percentage of cells viabilitywas more than 100% over the control demonstrated that stimulation of fibroblasts proliferation occurred. O-C 1% (6h) stimulated the proliferation more than O-C 5% (6h) ($P<0.05$). The rate of proliferationafter 5 days of treatment showed that, O-C 1% have the ability to induce fibroblasts proliferation *in-vitro* by approximately 60% over control and 40% over control for O-C 5%.

The effect of chitosan on the production of IL-8 was shown in Figure 3. The result demonstrated that, IL-8 secretion was induced in the supernatant of human dermal fibroblast treated with O-C 1% and 5%. IL-8 secretions were detected in both O-C 1% and 5% treated cells. In this study, we found that O-C 5% secreted higher percentage of IL-8 levels compared with O-C 1% ($P<0.05$). For O-C 1%, only low concentration induces the production of IL-8. Conversely, all concentration (6.25% to 100%) of O-C 5% induces the production of IL-8. The percentage of IL-8 levels increased with the reduction of test materials concentration. The result also demonstrated that fibroblasts treated cells secreted 2.3%, 17.7%, 13.7%, 26.5% and 50.6% (over negative control) of IL-8 with the reduction of test materials respectively. However, small amount of TGF-β was detected in all fibroblasts treated cells (result not shown). Untreated cells, triton-X treated cells and background control also expressed almost similar amount of TGF-β.

Discussion

Oligo chitosan derivatives tested was found did not induce cytotoxic effect to fibroblasts as confirmed by MTT reduction assay. The result shows that oligo chitosan exhibit high percentage of viable cells and maintained normal morphology of cell monolayer after three days of direct exposure to test materials. A slight change in culture medium was detected in the presence of material's leachable chemicals, but minimal changes did not affected fibroblasts growth. MTT test revealed that time exposure to test materials was not significant in relation to the cell growth as they constantly induced high number of viable cells at all time exposure. Direct contact test demonstrated that, O-C 1% derivatives exhibit high percentage of cell viability compared to O-C 5% derivatives. However all chitosan tested maintained high percentage of viable cells which exhibit more

than 80% of viability at all time of exposure. Cytotoxicity test using material extract technique also showed a comparable result. Studies by Lim C.K *et al.,* (2007) and AbdullRasad M.S.B., *et al.,* (2010) also showed that chitosan derivatives had the ideal properties of biocompatible materials tested on primary normal human epidermal fibroblasts and keratinocytes.

This study also confirmed that oligochitosan have the ability to induce the proliferation of human dermal fibroblasts which is an index for their potential used as wound healing agent. Fibroblasts are one of the important cells involved in healing process. Early in granulation tissue, fibroblasts deposit a matrix of fibronectin and hyaluronic acid which promote cell migration and proliferation (Hakvoort, 1999; Ueno *et al.,* 2001). The results demonstrated that low molecular weight with high deacetylation level of newly developed oligo chitosan derivatives induced the proliferation of fibroblasts. However, high molecular weight of oligo chitosan derivatives film did not directly enhance the rate of primary human dermal fibroblasts proliferation. In addition, high concentration of chitosan derivatives extract showed a reduction in the rate of cells proliferation. This is in accordance with Chen *et al.,*(2002), who reported that low molecular weight of carboxymethyl chitosan had a significant effect on growth promotion of normal skin fibroblasts and growth inhibition of keloid fibroblasts.

Fibroblasts treated with low molecular weight of oligochitosan demonstrated the stimulation of fibroblasts proliferation approximately 50% over control. Stimulation of cells proliferation was elevated at high concentration of chitosan derivatives extract. Nevertheless, a slight reduction in the rate of proliferation was observed at 100% of chitosan extract due to decreasing of dehydrogenase enzyme production by metabolically active growing cells. The results also verified that the rate of cells proliferation increased with time exposure revealed that O-C 1% and O-C 5% derivatives stimulate the proliferation and did not cause any toxicity effect on fibroblasts cell growth. Similar finding was reported by Mao *et al.*, (2004) which demonstrated that higher degree of deacethylation of chitosan membrane exhibited higher cell adhesion.

Chen *et al.,* (2002) and Howling *et al.,* (2001) revealed that chitosan samples with higher levels of deacetylation and lower molecular weight form stimulated fibroblast proliferation. Thus, the results clearly demonstrated that low molecular weight of oligo chitosan derivatives were more biologically active and therefore have more potential as wound healing agent or as a dressing material. However, the function and precise mechanisms of actions of chitosan derivatives on wound healing are not fully known yet. Additional research on oligo chitosan and its activities is needed for the application of biomaterials.

Chitin and its derivatives also have been shown to have an acceleratory effect on wound healing through induction of growth factors responsible in healing process. According to Abdull Rasad M.S.B. *et al,.* (2008) chitosan derivatives may play roles in the control of cell growth and proliferation of human fibroblasts. In this study, newly developed oligo chitosan induced the production of IL-8 secretion by fibroblasts. The induction of IL-8 production by fibroblasts suggested that chitosan derivatives used in this study could accelerate wound healing by enhancing the function of inflammatory cells and repairing cells. Mori *et al.,* (1997) also claimed that, chitin and its derivatives have been shown to possess positive effects on the wound healing process by inducing the production of different types of cytokines including IL-8, a cytokine that recruits neutrophils to the wounded site as well as stimulates vascularization (Rennekampff *et al.,* 2000).

However, oligo chitosan did not enhance the production of TGF-β by fibroblasts *in-vitro*. The result shows that small amount of TGF-β was detected in all treated fibroblasts. Nevertheless, untreated cells which serve as the positive control and background control also express almost similar amount of TGF-β. Thus, from this data we suggest that chitosan treated on fibroblasts did not enhance the expression of TGF-β. Expression of TGF-β in this study may be derived from the serum that has been supplemented in culture medium.

According to Wang *et al*. (1998), TGF-β is expressed to some degree in nearly all tissues. Transforming Growth Factorβ1 also known to be produced by macrophages and wounded fibroblasts especially during the granulation tissue formation and epithelialization. However, in normal skin, the expression of TGF-β1 gene and protein was very low (Jagadeesan and Bayat, 2006). In this study, the experiment had been done outside living organism and normal cascade of healing response was excluded. Since the expression of TGF-β cannot be detected from fibroblasts culture, we suggested that the expression of TGF-β may associate with other cells that act as activation factor. Without the presence of monocyte and leukocytes which serve as a main source of cytokine in healing response, TGF-β cannot be expressed.

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