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Induction of Permeability and Apoptosis in Colon Cancer Cell Line with Chitosan

ZAKIR HOSSAIN[1]* AND KORETARO TAKAHASHI[2]

[1.] *Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[2.] *Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan*

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ABSTRACT

The effect of chitosan on tight junctions (TJs) permeability on Caco-2 cell monolayer intestinal model was investigated. We have also investigated the effects of low molecular weight water-soluble chitosan (LMWC) on apoptosis in HT-29 cell. The changes in barrier properties of Caco-2 cell monolayers, including transepithelial electrical resistance (TEER) and permeability to lucifer yellow, were assessed in response to chitosan treatment. High molecular weight chitosan (HMWC) was found to cause up to 50% dose-dependent reduction in TEER of Caco-2 cell monolayer without damage to the cell membrane under lower concentration. The effect of HMWC on TJs was confirmed by increased permeability of lucifer yellow when cells were treated with 0.001-0.0001% HMWC for 120 min compared to control cells. Results showed that HMWC did not affect the F-actin of cytoskeleton. LMWC was proven to be an antitumor compound as shown by inducing apoptosis as a function of DNA fragmentation. These results suggest that HMWC is an useful drug delivery agent in paracellular pathway and LMWC has potential value in colon cancer therapy.

Key words: Chitosan, Caco-2 cells, HT-29 cells, F-actin, tight junction, apoptosis

INTRODUCTION

Chitosan is derived from crustacean shells by *N*-deacetylation, the second most abundant naturally occurring polymer[1,2]. Crab and shrimp shells are widely used for preparing this polymer[3]. Chitosan has been applied in the food, agricultural and cosmetic industries due to its high availability, high biocompatibility, biodegradability and ease of chemical modification. Beside these industries, scientists identified its potential application in pharmaceutical sciences[4-6]. Chitosan is applied in several drug delivery systems due to lack of toxicity and transmucosal penetration enhancer properties[7]. It induces very little cellular toxicity[8,9] and is naturally biodegradable because it is metabolized by lysozymes both *in vitro* and *in vivo*[10]. In addition, chitosan is attracting much attention as a possible mediator of transepithelial drug delivery due to its ability to mediate increased transepithelial drug permeation both *in vitro*[11,12] and *in vivo*[13,14]. Additionally, this polymer has been reported to reduce fat absorption in the intestine by binding fatty acids, triglycerides and bile acids and increasing their excretion. It is believed that by reducing the recycling of bile acids to the liver, chitosan induces hepatic synthesis of new bile constituents from cholesterol, thus contributing to reduction of cholestrolemia[15]. Furthermore, the residence time of the drug carrier systems in the gastrointestinal tract is an important factor controlling the bioavailability of drugs of high molecular weight. The mucoadhesive function of chitosan has received much attention for prolonging the residence time of dosage forms at the absorption site[16].

Despite the neoplastic origin of Caco-2 cells, these cells are able to differentiate spontaneously to mature entrocytes after 15-21 days of culture and express the brush border enzymes. Moreover, when cultured under specific conditions, they display the properties of an epithelial barrier[17]. Epithelial membrane provides a significant barrier to the free diffusion of substances. The presence of tight junctions (TJs) between neighboring epithelial cells prevents the free diffusion of hydrophilic molecules across the epithelium by the paracellular route[18,19]. The TJs consist of a complex of trans-membrane (e.g. occludin, claudin, JAM) and cytoplasmic (e.g. ZO-1, ZO-2, ZO-3, cingulin) proteins and are dynamically regulated by complex physiological mechanisms[20,21]. The confluent monolayers of Caco-2 cells mimic the epithelial barrier and display TJs that are impermeable to many polar drug compounds and are used as an *in vitro* model. The barrier integrity of these monolayers can be measured directly by measuring the transepithelial electrical resistance (TEER). The

* Author for correspondence. Tel: +81-75-712-9402;
Fax: +81-75-753-6233; Email: zakirh1000@yahoo.com

intestinal cells themselves are able to show an inflammatory response to injury[22]. The parameters of lactate dehydrogenase (LDH) release, an index of cell necrosis, would be convenient for the evaluation of damage and the protective effect of chitosan. There is evidence for an acute phase response in inducing apoptosis pathway[23]. Additionally, ogligochitosan induces apoptosis in neoplastic cells[22,24]. In view of the significance of chitosan and oligochitosan, the current study was designed to explore the TEER decreasing effect of chitosan. First, we measured the lucifer yellow flux through paracellular routes, % LDH release and F-actin alteration. Also, we determined cell viability and apoptosis.

MATERIALS AND METHODS

I. *Materials*

Human colon cancer cell lines Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB-38) were obtained from American Type Culture Collection (Rockville, CT, USA). Minimum essential medium (MEM), RMPI 1640 medium and Dulbecco's modified eagle medium (DMEM) were obtained from GIBCO (Grand Island, NY, USA). Fetal bovine serum (FBS), fetal calf serum (FCS) and L-glutamine were obtained from ICN Biomedicals Inc. (Costa Mesa, CA, USA). Chitosan CTA-4 (degree of deacetylation = 85%, average molecular weight = ~ 150,000, and specific viscosity = 14 cP) was generous gift from Katakura Chikkarin Co. Ltd. (Tokyo, Japan). Morpholinoethanesulfonic acid (MES) and rhodamine-labeled phalloidin were provided by Sigma Chemicals Company (St. Louis, MO, USA). Lucifer yellow (LY) CH lithium salt was purchased from Molecular Probe (Eugene, OR, USA). Sodium 2-(4-iodophenyl)-3-(4- nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium (WST-1), 1-methoxy-5-methyphenazinium methylsulfate (1-methoxy PMS) and *N*-2-hydroxyethyl poperazine-*N*-4-butanesulfonic acid (HEPES) were obtained from Dojindo Laboratories (Kumamoto, Japan). Cycloheximide was purchased from Tocris Cookson Ltd. (Avonmouth, UK).

II. *Cell Culture*

Caco-2 cells were grown in MEM with 26.2 mM sodium bicarbonate, 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, and 10% heat-incubated FBS. HT-29 cells were cultured in RPMI 1640 supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 1% nonessential amino acids, and 10% heat-incubated FBS. The cells were grown in 25-cm^2 flasks, and routinely subcultured every three-days at a concentration of 5×10^4 cells/mL. Cell cultures were maintained in a humidified incubator of 95% air -5% CO_2 atmosphere at 37°C.

III. *Preparation of Chitosan Solution*

First, chitosan was dissolved in acetate buffer solution (pH 4.4). Concentration of chitosan was 1% (w/v) and pH was adjusted at 6.0. Chitosan solution was stored at -20°C. Prior to use during experimentation, the solution was diluted in the appropriate concentration in Hank's balanced salt solution (HBSS: 137 mM NaCl, 5.36 mM KCl, 0.44 mM KH_2PO_4, 1.00 mM $CaCl_2$, 1.00 mM $MgCl_2$, 5.60 mM glucose, 0.30 mM Na_2HPO_4, 0.40 mM $MgSO_4$, 4.16 mM $NaHCO_3$) at pH 6.0.

IV. *Preparation of LMWC*

Low molecular weight water-soluble chitosan (LMWC) was prepared based on the method of Harish Prashanth and Tharanathan[25] with slight modifications. one percent chitosan solution was taken in a flask and, purged with N_2 stream at 60°C under stirring. Subsequently, potassium persulfate (0.8 mM) was added to the solution and the reaction was completed in 2 h. The reaction mixture was precipitated with ethanol to get LMWC, re-dissolved in double distilled water, dialyzed using 12 k-Da cutoff dialysis membrane (Viskase Companies Inc., Tokyo, Japan) overnight and lyophilized. The average molecular weight of prepared LMWC was 37000-40000 Da and degree of deacetylation around 80%[25].

V. *Permeability of the Caco-2 Cell Monolayer*

For transport studies, Caco-2 cells were plated at a density of 2×10^5 cells/ cm^2 onto a 12 mm polycarbonate transwell filter insert with 0.4-μm pores and a surface area of 1 cm^2 (Millipore Corporation Ltd., Bedford, MA, USA). The filter was placed in 24-well cell culture plates. This procedure was described in our previous report[26]. In brief, cells were grown 21 days after confluence for the experiments. Different concentrations of chitosan and 100 μM LY were applied to the apical (AP) chamber of Caco-2 cell monolayer. After incubation at 37°C for 2 h, the HBSS of basolateral (BL) chamber was collected and the LY concentration was determined by measuring fluorescent intensity with a Hitachi spectrophotometer F-2000 (Ibaraki, Japan). The excitation and emission wavelengths were 430 and 540 nm respectively.

VI. *TEER Measurement*

The TEER was measured using a Millicell(R) ERS (Millipore Co., Bedford, MA, USA). The average TEER in the present experiment was 653 ± 38.4 ohm/ cm^2. TEER measures were started during the pre-equilibration time in HBSS and continued during the treatment, recording value every 40 min over 2 h of experimentation. Control filters maintained in HBSS for 2 h, displayed a stable baseline of TEER values. In addition, test solution was removed from the cells, washed twice with PBS and

replaced with DMEM in the AP chamber at pH 6.0 and BL chamber at pH 7.4 to monitor recovery. TEER was monitored in the next 48 h. TEER data were expressed as percentage of initial values at each time point.

VII. *Protein Synthesis Inhibition*

The protein synthesis inhibition experiment was performed according to the method of Dodane *et al*.[11]. In brief, protein synthesis inhibition studies were performed on differentiated Caco-2 cells treated with chitosan for 1 h on the apical side. Following removal of HMWC, HBSS at pH 6.0 was used to wash the inserts twice and incubated the cells with or without 2 μM cycloheximide (Tocris, UK) during recovery. For these experiments, an additional control was used to monitor the effects of cycloheximide, alone which consisted of three samples treated in the same manner as the experimental culture recovering in 2 μM cycloheximide.

VIII. *Determination of Cellular F-Actin*

The relative content of F-actin was determined by fluorescent phalloidin-binding assay as described by Dadabay *et al*.[27] with some modifications. Caco-2 cells were plated at 5×10^4 cells/mL in 24-well plates for 3 days. The cells were rinsed with PBS and then incubated for 30 min with or without HMWC. The cells were fixed with acetone/methanol (1:1, v/v) for 15 min and permeabilized with 0.2% Triton X -100 in PBS for 5 min. The fixed, permeabilized cells were incubated with 200 μL of 5.0 μM rhodamine-phalloidin in PBS for 15 min, which was sufficient to saturate the filamentous actin. The wells were washed three times with PBS and cells were extracted with 0.2 N NaOH. The fluorescence of each sample was determined by measuring the fluorescent intensity with a Hitachi F-2000 fluorescence spectrophotometer (Hitachi Co. Ltd., Ibaraki, Japan). The excitation-emission wavelength was 545-578 nm.

IX. *LDH Release Assay*

Measurements were performed using a LDH-Cytotoxicity Assay Kit (BioVision Research Products, Mountain View, CA, USA) following the manufacturer's instructions with slight modifications. Briefly, 200 μL of DMEM containing 1% FBS was collected from the AP chamber during different experimental periods, placed into a 96 well plate, incubated for 20 min in an incubator at 37°C and centrifuged for 20 min at 250 × g. A total of 100 μL of the supernatant was transferred into corresponding wells. A total of 100 μL of the reaction mixtures was added to each well and incubated for 30 min at room temperature in the dark. Absorbance of all samples was measured at 490-650 nm using a precision microplate reader (Molecular Devices, Sunnyvale, CA, USA). Percent cytotoxicity was calculated using following formula.

Cytotoxicity (%) = (Text sample – Low control)/(High control – Low control) × 100

Low control: 1×10^4 cells/well in 200 μL assay medium

High control: 1×10^4 cells/well in 200 μL assay medium containing 1% Triton X-100

X. *Assessment of Cell Viability*

HT-29 cells were cultured without any treatment into 96 well plates at 1×10^4 cells/mL in 200 μL of growth medium per well for 24 h. Cells were treated with different concentration of LMWC. Twenty microliter of WST-1 (3.3 mg/mL PBS including 7% 1-methoxy PMS) was added to each well containing 200 μL of medium with cells while plates were incubated in a 5% CO_2-humidified incubator at 37°C. The plates were incubated for 24, 48 and 72 h in the incubator at 37°C. Absorbance was measured at 450-650 nm on a precision microplate reader (Sunnyvale, CA, USA).

XI. *Enzyme-linked Immunosorbent Assay (ELISA) for Apoptosis*

DNA fragmentation was measured by a biotin-labeled anti-histone-antibody and a peroxidase conjugated anti-DNA antibody cell death detection ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). We described the methods in our previous report[28]. In brief, Caco-2 cells (2×10^3 cells/well) were plated in 96-well plates with 100 μL of medium and incubated for 24 h in the incubator. After 24 h incubation, cells were treated with chitosan for 24 h. The level of cytoplasmic histone-associated DNA fragments (mono and oligonucleosomes) in HT-29 cells was expressed as an enrichment factor, calculated using the following formula:

Enrichment factor = Absorbance of sample (treated)/Absorbance of corresponding control

XII. *Statistical Analysis*

Statistical analysis for comparison between groups was performed using SPSS statistical analysis package. Data was expressed as means ± standard deviations (SD). A difference was considered significant at P. The resulting P values for each group are indicated in the figures.

RESULTS

I. *Chitosan Decreased the TEER of Caco-2 Cells Monolayer*

Treatment of Caco-2 monolayers with 0.0001, 0.0005 and 0.001% HMWC solutions resulted in reductions in TEER values to 16, 20 and 50%, respectively after 2 h while 0.001, 0.01, and 0.1% LMWC solutions reduced in

TEER to 10, 17, and 25%, respectively (Figure 1). The TEER of control treated monolayers was not changed over the treatment time staying consistently at approx. 100% of initial baseline (time=0) TEER values.

II. *Chitosan Increases Tight Junction Permeability*

The LY flux across the Caco-2 cells monolayers at pH of 6.0 is depicted in Figure 2 after incubation with HMWC and LMWC. TEER values were measured every 20 min during the permeability experiment (data not shown). Paracellular permeation of LY significantly increased at a dose of 0.001% HMWC; but the increase was much smaller at a dose of 0.0001% HMWC at 2 h (Figure 2A). However, permeation of LY did not increase significantly at the dose of 0.1% LMWC at 2 h (Figure 2B).

III. *Recovery of Chitosan-Exposed Caco-2 Monolayers and Protein Synthesis*

The TEER of control monolayers did not changed significantly from baseline TEER in 48 h. 0.0001% HMWC-treated monolayers fully recovered their barrier properties by 24 h as assessed by TEER and 0.001% HMWC-treated monolayers recovered over 80% of their baseline resistance values within 48 h (Figure 3). No significant difference was observed in the recovery of Caco-2 cells treated with 0.001 HMWC with or without

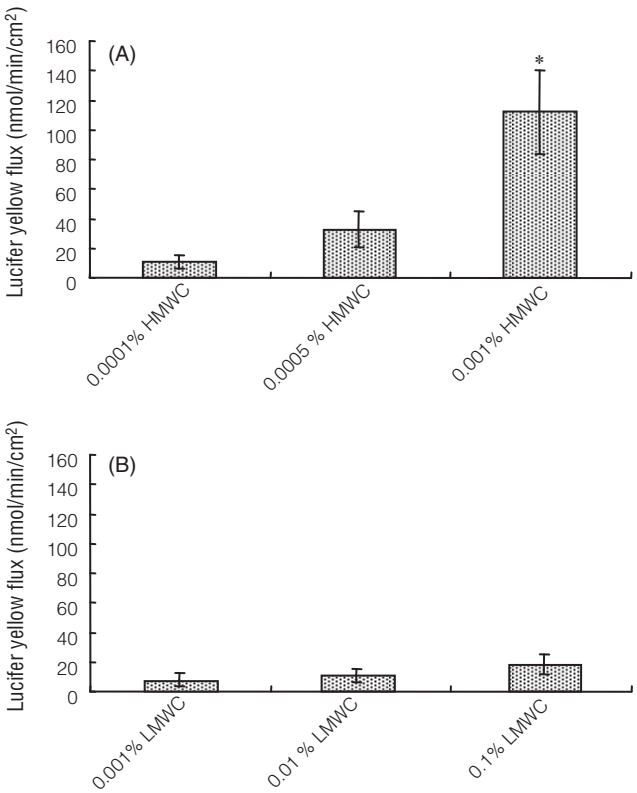

Figure 2. Lucifer yellow (LY) flux of cultured Caco-2 monolayer during HMWC (A) and LMWC (B) treatment. Lucifer yellow (100 mM) was added to the apical chamber and incubated for 2 h. LY was measured by fluorescence intensity at excitation and emission wavelengths of 439 and 540 nm, respectively. Results are means ± SD (n = 4). Asterisk above the error bars indicate significant difference between the treatment. HMWC: 0.0001 % versus 0.001% ($P < 0.01$).

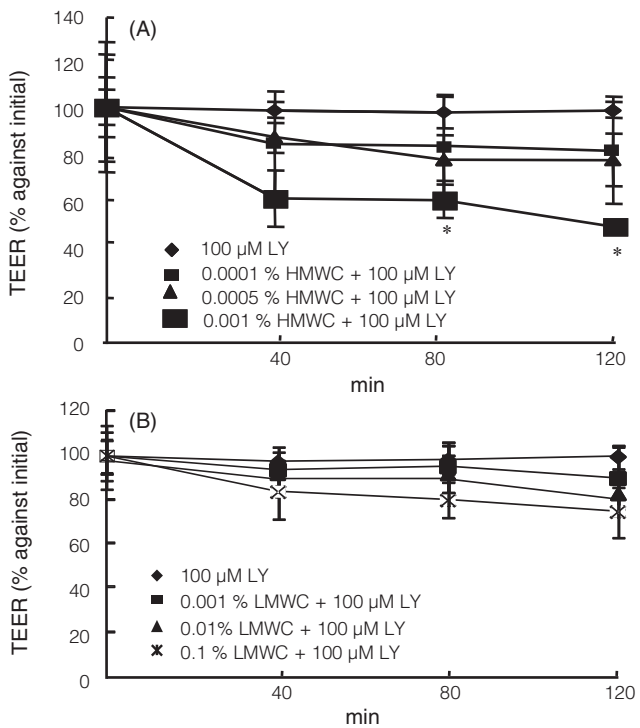

Figure 1. Effect of different concentrations of HMWC (A) and LMWC (B) on TEER of Caco-2 cells grown on filter insert. TEER values were calculated as the percentage of initial TEER values at each time point. Results are means ± SD (n = 4). Asterisk indicates significant difference from control ($P < 0.01$).

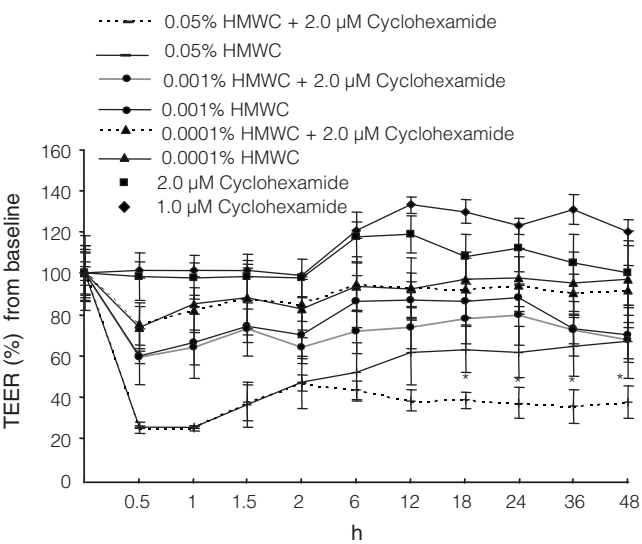

Figure 3. TEER recovery in the presence or absence of protein synthesis inhibitor, cycloheximide. Results are means ± SD (n = 4). Asterisk above the error bars indicates significant difference of recovery as compared to experiments without cycloheximide ($P < 0.01$).

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cycloheximide. However, protein synthesis was required after treatment with 0.05% HMWC. With the treatment of 0.001 and 0.05% HMWC, slightly increased alteration of the Caco-2 cells membrane by HMWC was observed (Figure 3).

IV. *Chitosan did not Reduce Staining of F-Actin*

We compared the effects of different doses of HMWC on the staining of F-actin by specific staining with fluorescent phalloidin. Control and HMWC treated cells exhibited the same staining of actin filament over the cell monolayer. Treatment with higher concentration of HMWC for 30 min did not reduce staining of F-actin (Figure 4).

V. *LDH Release*

The release of LDH from the Caco-2 cells to the extracellular medium after 12 h incubation with 0.001 or 0.0001 or 0.0005% chitosan was not significantly different from that of control. But significant releases occurred after 24 h incubation with 0.001% HMWC (Figure 5).

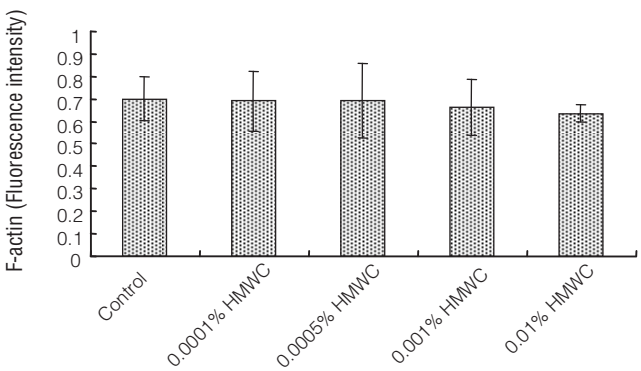

Figure 4. Effect of chitosan on F-actin.
F-actin was stained for 15 min with rhodamine-labeled phalloidin.

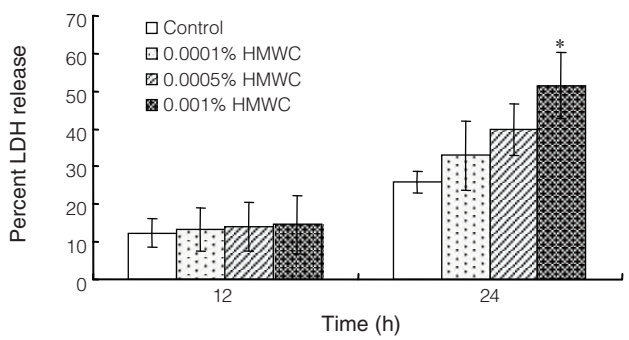

Figure 5. LDH release in the culture medium by Caco-2 cells due to the effect of chitosan at 24 h.
Caco-2 cells grown on the filter inserts were treated with different concentrations of chitosan. Percent LDH release was measured using a kit. Results are means ± SD (n = 5). Asterisks indicate significant differences from control (* $P < 0.01$).

VI. *Cell Viability*

The effects of LMWC on the growth of HT-29 are shown in Figure 6. Cell proliferation of HT-29 was reduced significantly as compared with control cultures after exposure to 200 μg/mL LMWC for 72 h, while HMWC had no growth inhibition on HT-29 (data not shown).

VII. *Apoptosis in Caco-2 Cells Induced by Chitosan*

Number of HT-29 cells decreased with LMWC. These results established the potent inhibition of growth of HT-29 cells. This observation might be caused by the induction of apoptosis in HT-29 cells. Hence, an attempt was made to find out whether LMWC really induced apoptosis in the HT-29 cells through nucleosomal DNA

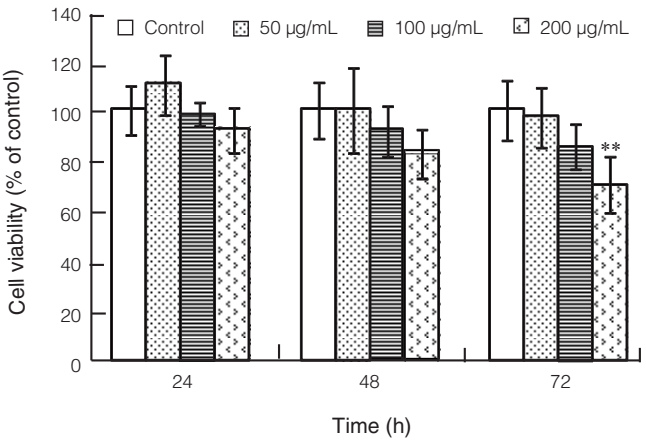

Figure 6. Effect of LMWC on the viability of colon cancer cells. Human colon cancer cells (HT-29) were treated with increasing concentrations of LMWC (50-200 μg/mL) for 1-3 days. Viable cells were determined by WST-1 dye reduction assay. Data the means ± SD (n = 8). ** $P < 0.01$ versus controls.

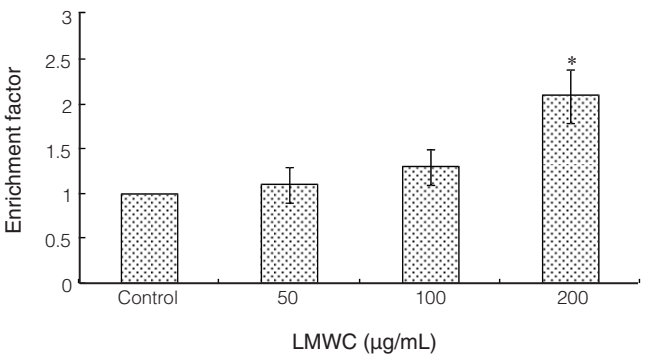

Figure 7. Chitosan (LMWC) induced apoptosis in HT-29 cells.
Cells were preincubated without any treatment for 24 h followed by treated with 50, 100 and 200 μM chitosan (LMWC) for 72 h. Apoptosis was quantified using an ELISA assay. Results are represented as relative fold increase in apoptosis as compared with control. The bars represent ± SD (n = 3). Significant difference compared with the control is indicated: * $P < 0.01$ versus control.

fragmentation. Enrichment factor increased with LMWC (Figure 7).

DISCUSSION

The extent of TEER response to concentrations of HMWC up to 0.001% was consistent in our current study. However, Ranaldi *et al.*[19] observed similar reductions in TEER (80%) with concentrations of HMWC as low as 0.0004%. Additionally, Holme *et al*.[29] reported the same results with 0.5% chitosan. This may be explained in part by the higher and lower molecular weight chitosan used by Ranaldi *et al*.[19] and Holme *et al*. [29] , respectively, compared with molecular weight of ~ 150,000 Da in our study. Another factor that appeared to affect the extent of response to chitosan was degree of deacetylation. The general consensus regarding this variable appears to be that a degree of deacetylation higher than 80% provides the greatest effect on cells in culture and is consistent among the majority of articles in this field.

At all concentrations tested, the achieved effects could be reversed after removal of the chitosan solutions. With removal of the chitosan solutions, repeated washing and substituting the fresh DMEM to the AP chamber again, cell monolayers started to return slowly to the initial values. However, because of the high viscosity and adhesive character of chitosan, probably not all the chitosan material could be removed from the cell surface without damaging the cells; therefore the reversibility observed was gradual. These results were in agreement with the results of Borchard *et al.*[18]. In addition, recovery was shown to require *de novo* protein synthesis only in the case of high concentrations of chitosan (0.5%) for 30 min[11]. In the current study, we found the protein synthesis was necessary for 0.05% chitosan at 4 h. The need for *de novo* protein synthesis to obtain a complete recovery was observed solely following 0.05% chitosan treatment.

Chitosan did not affect the actin ring of Caco-2 cells. This was consistent with results from studies by Dodane *et al.*[11] and Smith *et al.*[12] but slightly inconsistent with studies of Ranaldi *et al.*[19] and Artursson *et al.*[30] who demonstrated a decrease in fluorescent intensity of actin staining in Caco-2 cell actin ring after treatment with chitosan chloride and glutamate respectively. Dodane *et al.*[11] and Smith *et al.*[12] also reported shortening of the BL actin filaments and appearance of actin aggregates after treatment with chitosan. This finding was in agreement with the hypothesis that F-actin is directly or indirectly associated with the proteins in the tight junctions[31]. In principle chitosan is unlikely to enter cells due to its high molecular weight. Dodane *et al.*[11] reported that filaments of actin, a cytoskeletal protein, were visualized throughout the cytosol as expected in control cells. After incubation with 0.1% chitosan, a pattern of occludin thickened at the cell periphery and a decrease in fluorecent intensity in some areas was observed. We anticipated that alteration of TJs proteins (ZO and occludin) might be related on the effect of chitosan on TJs permeability. Another possible mechanism could be that the divalent cations may interact with putative binding site either on the chitosan itself or on the cell membrane and increase the TJs permeability[32].

Minimal disruption of the plasma membrane indicated that chitosan is less toxic to the Caco-2 cells. Significant release of LDH, a parameter frequently used to access membrane damage with 0.001% chitosan, was observed for 24 h in our present study. In fact LDH content in the extracellular medium did not increase even when the cells were exposed to 0.001% chitosan for 12 h. It was demonstrated that within 40 min of 0.5% chitosan treatment, all cells displayed nuclear labels, indicating a slight alteration of the epithelial membrane by chitosan[33].

We have shown that LMWC increased the apoptosis in human colon adenocarcinoma, HT-29 cells. Our results concurred with the results of Hwang *et al.*[34] who found that increasing concentrations of chitosan inhibited the growth of the mouse monocyte-macrophage, RAW 264.7 cell line. In addition, Yamada and Clarke[35] attempted to find out whether chitosan induce apoptosis in the Ehrlich ascites tumor (EAT) cells through nucleosomal DNA fragmentation. The *in vivo* effect of chitosan on the formation of ascites in EAT-bearing mice showed a significant decrease in the volume of ascites and, in treated mice as compared to control mice[25]. The 24-h incubation with chitosan produced a 25% increment of caspase-3 activity in Caco-2 cells compared to the untreated control[36]. Induction of apoptosis of bladder cancer cell line by chitosan[37] and suppression of gastric and colon cancer cell proliferation were reported[38]. These results were consistent with our findings that chitosan (LMWC) showed anticancer activity. We assume that cell cycle regulatory proteins might be one of the possible underlying mechanisms as DNA fragmentation was found with LMWC. In this report we found that alterations in tight junction permeability following treatment of Caco-2 cells with HMWC. We also found that LMWC induced apoptosis in HT-29 cells.

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