

Cytotoxicity and genotoxicity of chitooligosaccharides upon lymphocytes

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Two COS mixtures and a low molecular weight chitosan (LMWC) were tested for potential cytotoxicity and genotoxicity upon human lymphocytes. Genotoxicity was evaluated *in vitro* by cytokinesis-blocked micronucleus and alkaline comet assays, while cytotoxicity was assessed by flow cytometry analysis. Our results suggest that COS do not exhibit any genotoxicity upon human lymphocytes, independently of MW or concentration. However, above 0.07 mg/mL COS induced strong cytotoxic effects. According to the concentration used, such cytotoxicity will induce cell death, essentially by necrosis (>0.10 mg/mL) and/or apoptosis (<0.10 mg/mL). The level of necrosis/apoptosis induced by high COS concentrations, suggests a promising use as apoptosis inducers in specific cancer situations.

1. Introduction

Chitosan, a biopolymer comprising glucosamine and *N*-acetylglucosamine residues, is an *N*-deacetylated product of chitin, as well as one of the most abundant polysaccharides in nature [1]. This cationic polysaccharide has been widely used in a variety of pharmacological and biomedical applications, besides as a dietary supplement, owing to its claimed biological properties (e.g. antioxidant, prebiotic, antimicrobial and cholesterol regulator), which might be used to human benefit [2]. However, its high molecular weight (MW), which hampers solubility in acid-free aqueous media, has limited its practical applications [3]. Recent studies pertaining to chitosan have focused on conversion thereof to water soluble oligosaccharides.

Chitooligosaccharides (COS) – depolymerized products of chitosan obtained by chemical or enzymatic hydrolysis, have recently attracted much attention as potential nutraceutical agents. These chitosan derivatives (generally, the MW of COS is 10 kDa or less) [4], also seem to possess several biological properties as prebiotic, antioxidant, antibacterial and anti-inflammatory among others [5–7]. Furthermore, their ready uptake by cells, namely intestine cells, makes theoretically possible for COS to be accessible to the

entire human body, enhancing the range of possible applications for COS [3].

Despite the extensive studies on the biological activities of chitosan and COS, there is no strong experimental evidence available regarding the biocompatibility of COS. *In vitro* and *in vivo* evaluations of chitosan toxicity have been reported elsewhere [1,8,9], and considered it as a biocompatible polymer. Yet, some studies also reported cell toxicity dose-dependent [10–12]. With regard to COS, the studies are even scarcer, mainly based on the MTT colorimetric assay, and reported contradictory conclusions: Rajapakse et al. reported the absence of toxic effects by COS, at 0.050–1.0 mg/mL upon human and mouse leukocyte cell lines [13], but Xu et al. claimed that at 0.80 mg/mL COS induces apoptosis upon human cells [14].

In view of the above, the main objective of this study was to evaluate the biocompatibility of COS by studying their cytotoxicity and/or their mutagenic potential, upon human lymphocyte cultures.

2. Materials and methods

2.1. Materials

Two COS mixtures, named COS3 and COS5, were purchased from Nicechem (Shanghai, China). Low MW chitosan (LMWC) was purchased from Sigma-Aldrich (Sintra, Portugal). All said compounds were derived from crab shells. The chemicals used in the

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experimental work were purchased from Sigma–Aldrich (Sintra, Portugal).

2.2. Characterization of chitooligosaccharides

The average MW of both COS mixtures was assessed by size exclusion chromatography (SEC). Two combined TSKGel series columns (G2500PWXL × G5000PWXL) together with a PWXL guard column were used, coupled with a RID-10A Shimadzu refractive index (RI) detector. A flow rate of 0.8 mL min⁻¹ and a mobile phase solution of 0.5 M AcOH–0.2 M AcONa at pH 4.4–4.5 (25 °C), were found to be the most suitable conditions to evaluate COS molecular weight. Pullulan (TOSOH Biosciences) of different molecular weights were used as standards to calibrate the column, and quantification of COS was performed by external calibration, using chitobiose as standard. Data provided by the SEC–HPLC system were collected and analyzed using the Chromeleon system version 6.7. The DD was determined using a FT-IR Perkin Elmer infrared spectrometer. An aliquot of COS sample was mixed with potassium bromide (1:1000) and compressed into pellets. The IR spectra were recorded and the absorbance values of the suitable absorption bands were calculated using the base line method. The DD was calculated from the value of the absorption band ratio $A_{\text{amide peak}}/A_{\text{reference peak}}$. A number of absorption band ratios have been proposed in the literature, differing either in the band selected as in the internal reference band. One such band ratio is A_{1655}/A_{3450} , determined using a line draw from 4000 cm⁻¹ to 2500 cm⁻¹ as the base line for the hydroxyl group band, and one drawn from 1800 cm⁻¹ to 1600 cm⁻¹ as the base line for the Amide I band [16]. The DD was thus calculated according to the following equation:

$$\text{DD}(\%) = 100 - \left(\frac{A_{1655}}{A_{3450}} \right) \times 115$$

This procedure has been found to give results in agreement with those given by dye absorption [15,16] for samples having a degree of *N*-acetylation within the range 0–55%.

2.3. Evaluation of genotoxicity by cytokinesis-blocked micronucleus assay

Fresh peripheral blood samples were collected from healthy volunteers, into heparinized vacutainers. The blood samples were suspended in RPMI 1640 culture medium, supplemented with 10% (w/v) fetal bovine serum (FBS), L-glutamine, and penicillin; phytohaemagglutinin, at 1% (w/v), was used as a mitogen to stimulate lymphocyte mitosis. In all sets of experiments ($n=3$), a negative control (whole blood in PBS) was used, as well as a positive control (using the mutagenic agent, cyclophosphamide, at 5 mg/mL). Either COS mixture and LMWC were tested at 4 different concentrations – 1.0, 0.1, 0.01 and 0.001 mg/mL. Duplicate cultures were set up for each experimental point, within 60 min after venipuncture. Cytochalasin B, an inhibitor of the mitotic spindle that prevents cytokinesis, was added (5 mg/mL) at 44 h of incubation; blood cell cultures were incubated at 37 °C for 72 h after experiment initiation. The lymphocytes were then isolated from the other blood cells by density gradient separation (Histopaque-1077 and -1119) and additional 2 washing steps were performed with 3% (v/v) FBS saline solution with. The lymphocytes were fixed in 3:1 methanol/glacial acetic acid, dropped onto clean microscopic slides, air-dried and stained with Wright stain. For each sample, 1000 binucleated lymphocytes were blindly scored using a Leica light optical microscope (Wetzlar, Germany), following the scoring criteria outlined elsewhere [17]; the numbers of micronuclei, nucleoplasmic bridges and nuclear buds per 1000 binucleated lymphocytes, were recorded. The nucleous division index (NDI), a measure of the cell division kinetics, was scored in the same slides, according to the method

of Eastmond and Tucker [18]; accordingly, 500 viable cells were counted to determine the frequency of lymphocytes with 1, 2, 3 or 4 nuclei, and the NDI calculated using the formula:

$$\text{NDI} = \frac{M1 + 2M2 + 3M3 + 4M4}{N},$$

where $M1$ – $M4$ represent the number of lymphocytes with 1–4 nuclei, respectively, and N is the total number of viable cells scored (excluding necrotic and apoptotic cells).

2.4. Evaluation of genotoxicity by the comet alkaline assay

Fresh peripheral blood samples were collected and treated, as described above for cytokinesis-blocked micronucleus assay. Following the guidelines proposed by Tice et al. [19], 10 µL of treated or control lymphocytes (ca. 10⁴ cells) was added to 120 µL of 0.5% (w/v) low-melting point agarose at 37 °C, layered onto a pre-coated slide with 1.5% (w/v) regular agarose and, finally, covered with a coverslip. After brief agarose solidification under refrigerated conditions, the coverslip was removed, and the slides were immersed in a lysing solution – consisting of 2.5 M sodium chloride, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris–HCl buffer at pH 10 (Sigma–Aldrich), 1% (w/v) sodium sarcosinate (Sigma–Aldrich) with 1% (w/v) Triton X-100 (Sigma–Aldrich) and 10% (w/v) dymethylsulfoxide (DMSO). Just prior to electrophoresis, the slides were left for 20 min in an alkaline buffer containing 0.3 mM NaOH (Merck) and 1 mM EDTA (pH > 13); electrophoresis was then carried out for 20 min, at 25 V (0.86 V/cm) and 300 mA. Afterwards, the slides were neutralized in 0.4 M Tris–HCl (pH = 7.5), fixed in absolute ethanol and stored at room temperature, until analysis. In order to minimize extraneous DNA damage caused by ambient ultraviolet radiation, all steps were performed with reduced illumination.

2.5. Evaluation of cytotoxicity by flow cytometry

Fresh peripheral blood samples were collected from healthy volunteers, into heparinized vacutainers. Lymphocytes were then isolated by density gradient separation (Histopaque-1077 and -1119). Three extra washes with a cold saline solution containing 3% (w/v) FBS were performed. The viability of the lymphocytes was evaluated by the trypan blue exclusion test, using a Neubauer counting chamber. Lymphocytes were then resuspended, at a concentration of 1 × 10⁶ viable cells/mL, in RPMI 1640 culture medium, supplemented with 10% (w/v) FBS, L-glutamine and penicillin. In all sets of experiments ($n=3$), a negative control (with PBS) was used, as well as a positive control with staurosporine at 4 µM – a strong cytotoxic alkaloid added 8 h before the end of incubation. Either COS mixtures and LMWC were added to the lymphocyte suspensions at the 4 concentrations used in the other assays – 1.0, 0.1, 0.01 and 0.001 mg/mL, and incubated for 24 h at 37 °C. The assay was performed in a 96-well plate, and each tested condition had 4 replicates. After incubation, the cells were washed twice with a cold saline solution, and then stained according to the general Annexin V staining procedure by BD Biosciences (Annexin V-PE Apoptosis detection kit I, BD Biosciences, San Diego, US): the cells were resuspended in 1 × binding buffer, to obtain a cell density of ca. 10⁵ cells; Annexin V and 7-Aminoactinomycin D (7AAD) were then added, and the samples incubated for 15 min at room temperature, in the dark; 400 µL of 1 × binding buffer was finally added to each tube. The treated samples and controls were analyzed by flow cytometry within a 1 h period [20,21]. Flow cytometric analysis was carried out in a FACS Calibur (San Jose, CA, USA) based on the acquisition of 20,000 events. Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, whereas logarithmic detectors were used for all three fluorescence channels

Table 1

Major characteristics (average or average \pm standard deviation) of COS3, COS5 and LMWC, determined by SEC and FTIR.

Compound	MW (kDa)	DD (%)
COS3	1.763 \pm 0.7	64.14 \pm 1.96
COS5	4.134 \pm 0.6	66.24 \pm 0.48
LMWC	125.6 \pm 4.2	70.23 \pm 0.93

(FL-1, FL-2 and FL-3). Compensation for spectral overlap between FL channels was performed for each experiment using single-color-stained cell populations of positive control. All data were collected ungated to disk and were analyzed using CELLQuest Pro software. Lymphocytes were then analyzed for their expression of Annexin and 7AAD to determine the number of viable cells: Annexin V and 7AAD negative (Annexin V⁻/7AAD⁻); cells undergoing apoptosis, Annexin V positive and 7AAD negative (Annexin V⁺/7AAD⁻); and dead cells or cells that were in latest stage of apoptosis, Annexin and 7-AAD positive (Annexin V⁺/7AAD⁺).

2.6. Confocal analysis

FITC-labeled COS was synthesized by adding 100 ml of dehydrated methanol followed by 50 ml of FITC in methanol (2.0 mg/ml) to 100 ml of COS (10.0 mg/mL of water) in the dark at ambient temperature. After 3 h, the labeled polymer was precipitated in 0.2 M NaOH. After centrifugation of the precipitated product at 6000 rpm for 10 min, the supernatant solution was discarded to recover the product. Five ml of Milli-Q water with a few drops of 1 N HCl was then added and the product was dissolved again. This purification process was repeated five times until fluorescence from FITC could not be detected in the supernatant. The FITC-labeled COS was then freeze-dried. Following a procedure similar to the evaluation of cytotoxicity, FITC labeled COS was added to the lymphocyte suspensions at 0.1 mg/mL, and incubated for 8 h at 37 °C, in the dark. After incubation, the cells were washed twice with a cold saline solution, and then were resuspended to obtain a cell density of ca. 105 cells. The lymphocytes were then dropped onto glass, stained with DAPI (250 μ L of a 300 nM solution, for 10 min) and with Alexa Fluor 568 (500 μ L of a 1 μ g/mL solution, for 10 min), and finally mounted in Vectashield (Vector Laboratories, Peterborough, UK). The samples were examined under an inverted confocal microscope (CLSM, Zeiss Axiovert 200M, Oberkochen, Germany) equipped with a LSM 5 Image Browser (Carl Zeiss, Oberkochen, Germany).

2.7. Statistical analyses

Mean values and standard deviations were calculated from the experimental data obtained, and analysis of variance (ANOVA) was applied to a 5% level of significance, using compound concentration and MW as main factors. Pairwise comparisons were done using the Bonferroni test, at the same level of significance. All statistical analyses were performed using the SPSS package program version 16.0.

3. Results

The major characteristics of the COS mixtures and LMWC are listed in Table 1.

The potential genotoxic effect of COS3, COS5 and LMWC on lymphocytes was determined by the cytokinesis-blocked micronucleus assay (Table 2). No significant differences on micronucleus-forming activity as a function of concentration or MW were observed ($P > 0.05$). Micronuclei were scored in populations of 1000 binucleated lymphocytes, either as micronucleated binucleated

Table 2

Results of the cytokinesis-blocked micronucleus assay (average or average \pm standard deviation), for COS3, COS5 and LMWC, at several concentrations.

MW	Concentration (mg/mL)	MN/1000 binucleated cells	NDI
C (–)	–	3.00 \pm 0.71	1.53
C (+)	5.0	14.50 \pm 2.12 [*]	1.38
COS3	1.0	3.00 \pm 1.41	1.30
	0.1	2.50 \pm 0.71	1.42
	0.01	1.50 \pm 0.71	1.51
	0.001	2.50 \pm 0.71	1.50
COS5	1.0	2.50 \pm 0.71	1.29
	0.1	2.50 \pm 0.71	1.40
	0.01	2.00 \pm 0.00	1.49
	0.001	3.50 \pm 0.71	1.52
LMWC	1.0	2.50 \pm 2.12	1.43
	0.1	3.50 \pm 0.71	1.47
	0.01	1.50 \pm 2.12	1.48
	0.001	2.50 \pm 0.71	1.48

NDI: nucleous division index; MN: micronucleus.

^{*} $P < 0.05$.

cells, or as total number of micronuclei. The use of cyclophosphamide (positive control) induced almost a 5-fold increase in the micronucleus-forming activity, as compared to the negative control.

Values for the NDI are also depicted in Table 2. This cell division kinetics index presented significant differences dependent on concentration and MW: COS3 and COS5, when at 1.0 mg/mL, lead to significant lower NDI values than those observed for the other COS concentrations, and also observed for the negative control and for all the LMWC concentrations tested; however, such NDI values produced by 1.0 mg/mL COS3 and COS5, were similar to those presented by the positive control.

The results for the comet assay are shown in Table 3. The comet tail length (CTL) and tail moment (CTM) were estimated for each concentration, based on populations of 100 lymphocytes, since it is widely accepted that CTL and CTM are directly proportional to the extent of DNA damage [22,23]. Analysis of variance of CTL and CTM did not reveal significant differences ($P > 0.05$) between negative control and lymphocytes treated with COS/LMWC. However, both CTL and CTM of lymphocytes treated with cyclophosphamide (positive control) were significantly higher than those of the negative control, and those with COS/LMWC.

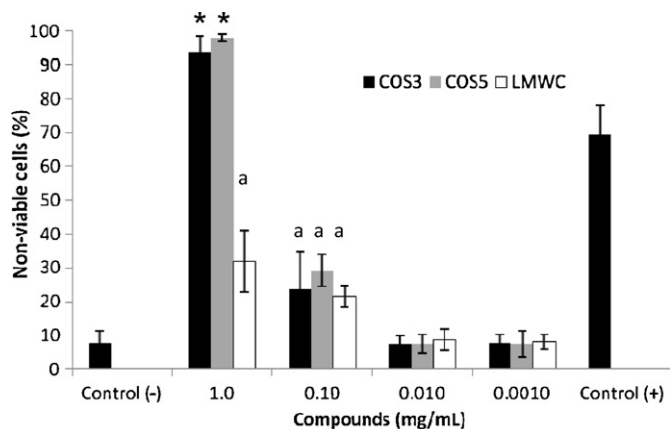
Lymphocytes treated with high concentrations of COS, showed a significant increase in non-viable cells when compared with the negative control (Fig. 1); at 1.0 mg/mL the percentage of non-viable

Table 3

Results of the comet alkaline assay (average or average \pm standard deviation), for COS3, COS5 and LMWC, at several concentrations.

MW	Concentration (mg/mL)	Tail length (μ m)	Tail moment (%)
C (–)	–	41.02 \pm 5.32	0.612
C (+)	5.0	103.3 \pm 18.1 [*]	9.58 [*]
COS3	1.0	43.19 \pm 7.41	0.696
	0.1	42.86 \pm 4.08	0.640
	0.01	40.15 \pm 6.35	0.612
	0.001	40.32 \pm 2.28	0.612
COS5	1.0	44.08 \pm 7.07	0.702
	0.1	42.57 \pm 7.14	0.648
	0.01	40.35 \pm 5.14	0.611
	0.001	39.86 \pm 2.12	0.608
LMWC	1.0	41.63 \pm 5.12	0.623
	0.1	40.25 \pm 3.35	0.607
	0.01	40.79 \pm 2.87	0.612
	0.001	40.74 \pm 3.92	0.611

^{*} $P < 0.05$.



* $P < 0.01$ (versus negative control)

^a $P < 0.05$ (versus negative control)

^b $P < 0.01$ (versus COS3 and COS5 at 1.0 mg/mL)

Fig. 1. Non-viable lymphocytes (Annexin V⁺/7AAD⁻ plus Annexin V⁺/7AAD⁺), following treatment with COS3, COS5 or LMWC for 24 h, at 37 °C (average \pm standard deviation).

cells were above 90.0%, and were even higher than those exhibited by the positive control, ca. 70% (treated with staurosporine); lymphocytes treated with 1.0 mg/mL of LMWC, showed a significant lower fraction of dead cells (ca. 30%) compared to COS3 and COS5, and to the positive control; lower concentrations (0.01 and 0.001 mg/mL) of COS and LMWC led to results similar to those presented by the negative control (i.e. <10%).

Further analyses with flow cytometry were performed, in order to follow the percentage of apoptotic and necrotic cells present in the lymphocyte populations treated with COS3 and COS5, at concentrations similar or higher than 0.01 mg/mL – above which we observed changes in the viability of the lymphocytes (Fig. 2). The extent of apoptosis in cells treated with staurosporine was about 60.1%; this apoptosis value was significantly higher than that induced by COS at any concentration tested ($P < 0.05$). Above 0.50 mg/mL, both COS induced necrosis higher than 70%, and apoptosis below 10% (data not shown). As COS concentration was decreased, the necrosis rate also decreased; in contrast, apoptosis started to increase until a maxima of 22.1% (for COS3 at 0.20 mg/mL) or of 34.7% (for COS5 at 0.30 mg/mL); at these concentrations, the necrosis values are statistically higher than the negative and positive controls ($P < 0.05$). Although the percentage of non-viable cells

were approximately the same for either COS at the same concentration ($P > 0.05$), the level of apoptosis was always higher in the case of COS5. Conversely, the necrosis percentages were higher for COS3 (except at 0.50 mg/mL); below 0.070 mg/mL, the values became statistically identical between negative control and both COS mixtures.

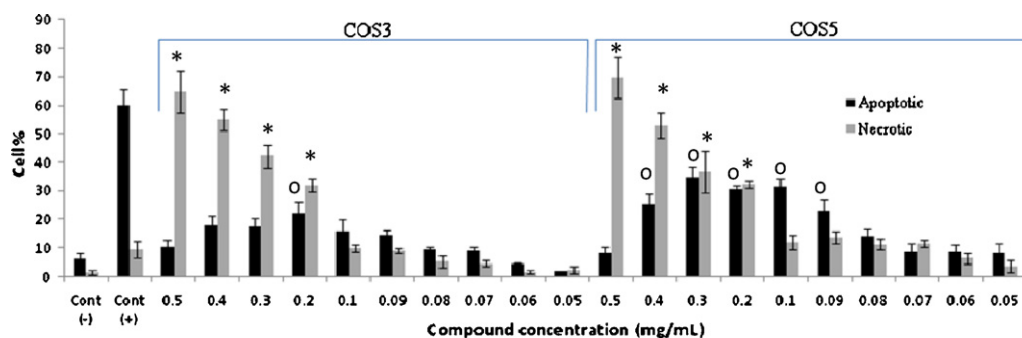
4. Discussion

In this research effort, we aimed to explore the relation between the MW and concentration, and toxicological effects, of two COS mixtures and a LMWC. Although chitosan has been the subject of intense studies and claimed to be a non-toxic biocompatible polymer in several reports [24–26], COS safety has not to date been comprehensively assessed in cytogenetic terms.

Biocompatibility of a compound refers to the extent to which its molecule does not have toxic effects or cause injury upon biological systems. To consider it as biocompatible, it is thus of great importance to submit the molecule under study to a number of pre-toxicity tests, *in vitro* or *in vivo* [25]. *In vitro* tests are faster and ethically less demanding, so they were selected here; in addition, they are usually more reproducible and sensitive, besides allowing cellular and molecular reactions to be handled outside the organism, in a simple manner.

The cytokinesis-blocked micronucleus assay is a genotoxicity test that provides simultaneous information on a variety of chromosomal damage endpoints that may reflect chromosomal loss, breakage and rearrangement, as well as gene amplification [27]. This assay has been routinely used in mutagen/carcinogen screening programs, to detect agents that cause chromosomal damage and spindle dysfunction [28]. Our results showed that COS and LMWC do not possess mutagenic potential at the studied concentrations, as they did not present differences in micronuclei frequency, as compared with the negative control ($P > 0.05$); indeed, as the micronucleus frequency induced by different concentrations and MW was essentially constant, we may say that the absence of genotoxic effects upon human lymphocytes of COS and LMWC appears to be MW- and dose-independent.

The NDI is useful to compare the mitogenic response of lymphocytes and the cytostatic effects of the agents under study, as it provides a measure of the proliferative status of the viable cell fraction, thus being also a useful biomarker of immune function [29]. The NDI values showed clearly that both COS mixtures, at 1.0 mg/mL, possess a cytostatic effect; since the NDI values (≤ 1.30) were considerably lower than the negative control, meaning that a major fraction (70%) of the viable lymphocytes failed to undergo cell division.



* $P < 0.05$ (versus negative and positive control)

^o $P < 0.05$ (versus negative control)

Fig. 2. Necrotic (Annexin V⁺/7AAD⁺) and apoptotic (Annexin V⁺/7AAD⁻) lymphocytes, following treatment with the two COS mixtures for 24 h, at 37 °C (average \pm standard deviation).

The comet assay constitutes an alternative approach to genotoxic studies, and we used it to confirm the absence of genotoxic effects by COS. In this test, cells exhibiting an increased frequency of DNA double strand breaks, display an increased rate of migration of DNA. In addition, due to the prevailing alkaline conditions, this test also offers enhanced sensitivity to identify genotoxic activity, which tends to induce more single strand breaks and alkali-labile sites than double strand breaks [19,30]. This assay has, indeed, been widely claimed to be more sensitive than CBMN (and Ames) test. Measurements of the tail length of released damaged DNA are described to correlate well with the mutagenic and carcinogenic properties of the compounds under study [25,31].

Our comet assay results confirmed the parallel CBMN assay results: COS and LMWC did not exhibit genotoxic effects upon human lymphocytes. Recall that the tail length – i.e. the distance of DNA migration from the body of the nuclear core, is used to evaluate the extent of DNA damage; on the other hand, the tail moment constitutes a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and of the number of relaxed/broken pieces (represented by the intensity of DNA in the tail). Both tail length and moment were not significantly affected by COS and LMWC compared with negative control. In contrast, the DNA damage in cells exposed to cyclophosphamide was significantly higher – the damaged DNA actually migrated almost 3-fold more than all the other tested samples. This absence of DNA damage is most likely related to the reported protective effects of COS upon damaged DNA [32]; however, such protective actions have not yet been fully elucidated. *In vivo* studies by Yoon et al., showed that COS did not affect the frequency of micronucleus or chromosomal aberrations in bone marrow cells, independently of the concentrations of COS [33].

A number of studies have meanwhile shown that chitosan induces apoptosis *in vitro* [34–36] and *in vivo* [37]; however, data regarding COS are essentially few and inconclusive. Flow cytometry can provide rapid, quantitative and objective evaluation of cell viability, and may further provide enumeration of apoptotic or necrotic cells. It has become the method of choice to assay for apoptosis and necrosis in a variety of cell systems. The double-staining Annexin V/7-AAD assay discriminates cells that are undergoing early or late apoptosis and necrosis [38]. Hence, we chose this method to provide a broader understanding of the cytotoxic effects of COS upon lymphocytes.

Our experimental data demonstrated that COS at high concentrations (1.0–0.1 mg/mL), independently of their MW, exerted strong cytotoxic effects against human lymphocytes – which were clearly dose-dependent. LMWC showed a significantly lower toxicity than either COS mixtures ($P < 0.01$) at high concentrations, thus suggesting that COS are much more toxic than chitosan. Due to the cationic nature of chitosans and COS, the surface charge of these molecules has been claimed as the major factor affecting said cytotoxic activity, owing to the electrostatic interaction between the negatively charged groups of the lymphocyte surface (i.e. glycoproteins) and the positively charged amino groups of chitosans and COS [39]. Since the DD is approximately the same for LMWC and both COS mixtures (Table 1), we may hypothesize that chain length plays a crucial role upon induction of cytotoxicity.

COS have previously been reported to be more reactive than LMWC [40]. Furthermore, as we have previously reported [41], high concentrations (i.e. 1.0–0.5 mg/mL) of COS induced oxidative stress on cells via a pro-oxidant effect, opposed to the effect produced by LMWC at the same concentration; and damaged cell membranes as well, via binding to its proteins [40]. The induction of oxidative stress when at high concentrations may be a possible mechanism of induction of toxicity by COS, as oxidative conditions have been widely reported as responsible for inducing cytotoxic effects upon human cells: Yang et al. showed that oxidative stress was the key

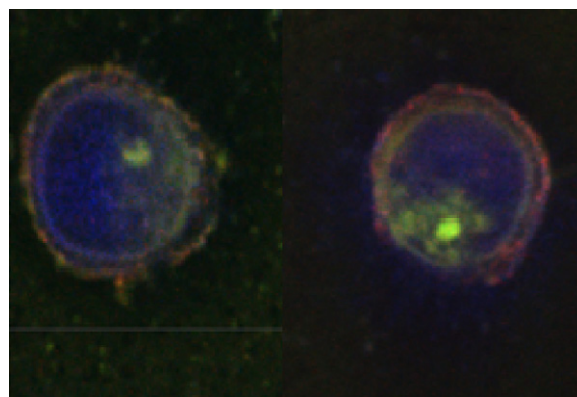


Fig. 3. Microphotograph of lymphocytes after 8 h incubation with FITC-labeled COS using a stain for DNA (blue – dapi) and a stain for cell membrane (red – Alexa Fluor 568). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

route cytotoxicity induction by several nanoparticles on fibroblasts cells [42]; Arakaki et al. reported the involvement of oxidative stress in tumor cytotoxic activity [43]; and Patlolla et al. described cytotoxicity, induced by potassium dichromate, upon HepG2 cells to be mediated by oxidative stress [44].

Another feature of COS that we have described [40], was their ability to induce changes in cell membranes. Confocal microscopy permitted us to conclude that COS did not just link to the lymphocyte membrane, but they were also able to penetrate the membrane and, eventually, bind to nucleolus (Fig. 3). On the contrary, LMWC could not enter directly into the cell, but merely interact with the cell membrane (data not shown).

Takimoto et al. have demonstrated that chitosan induces apoptosis via caspase-3 activation [36]. Our data indicated that LMWC induced cellular death (ca. 30% at 1.0 mg/mL) mainly by apoptosis as well. However, COS (when at high concentrations) seem to induce cell death, mainly, via necrosis (quick process, in which cells lose their membrane integrity and, thus, die rapidly as a result of cell lysis); when COS concentrations decrease (< 0.10 mg/mL), the primary mechanism of cell death switches to apoptosis. It has been documented that the mode of cell death may depend on the cell type, the type/concentration of stimulus, and environmental setting [45]; our results suggest that the mode of cell death varies with the concentration and the type (i.e. MW) of the chitosan derivative tested.

In summary, our experimental results suggest that chitosan oligomers do not exhibit any genotoxicity upon human lymphocytes, independently of MW or concentration. However, COS cannot be considered biocompatible molecules at levels above 0.07 mg/mL, since they appear to induce a strong cytotoxic effect upon the lymphocytes – concentration and chain length-dependent. According to the concentration used, such cytotoxicity will induce cell death, essentially via necrosis (> 0.10 mg/mL) or apoptosis (< 0.10 mg/mL). Below 0.07 mg/mL, neither COS nor LMWC produced toxic effects, suggesting that these molecules will be harmless to human cells under those conditions.

In any case, further studies are recommended, mainly *in vivo* tests, to eventually confirm these *in vitro* results, especially aiming at assuring COS and LMWC safe concentrations.

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