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## Anti-proliferation and radiosensitization effects of chitooligosaccharides on human lung cancer line HepG2

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## ABSTRACT

**Objective:** To observe the anti-proliferation and radiosensitization effect of chitooligosaccharides (COS) on human lung cancer cell line HepG2.

**Methods:** CCK-8 assay was employed to obtain the inhibition ratio of COS on HepG2 cells at 24 h after treatment. The clonogenic assay was used to analyze the cell viability of RAY group and RAY + COS group with X-ray of 0, 1, 2, 4, 6 and 8 Gy, and the cell survival curve was used to analyze the sensitization ratio of COS. Flow cytometry was employed to detect cell cycle and apoptosis rate in control group, RAY group and RAY + COS group after 24 h treatment.

**Results:** COS inhibited the proliferation of HepG2 cells, and the inhibition rate positively correlated with the concentration of COS. The cell viability decreased with increasing exposure dose in RAY group and RAY + COS group. The cell viabilities of RAY + COS group were lower than those of RAY group at the dose of 4, 6 and 8 Gy ( $P < 0.05$ ), and the sensitization ratio of COS was 1.19. There were higher percentage at G<sub>2</sub>/M phase and apoptosis rate, and lower percentage at S phase in RAY + COS group versus the other two groups ( $P < 0.01$ ).

**Conclusions:** COS can inhibit the proliferation of HepG2 cells, and enhance the radiosensitization of HepG2 cells, induce apoptosis and G<sub>2</sub>/M phase arrest.

## 1. Introduction

Despite of the rapid development of modern medical technology, cancer is still the biggest challenge in medical field, posing immense threat on human's life and health. Bad habits like smoking, together with worsening air pollution and industrial fumes directly cause the surging population with lung cancer [1,2]. Lung cancer has topped the list of cancer rate in many developed countries and morbidity in major cities of China, which urges the tough fight against lung cancer [3–5]. Radiotherapy is one of the three weapons against lung cancer and plays an immeasurably important role in the treatment of lung cancer with over 70% patients [6–8]. However, attacking wrong objects happens due to low capacity of radioactive rays

to identify the pathological cells, thus, the top priority for researchers is to efficiently eliminate the ‘invaders’ and meanwhile protect the ‘peers’. Chitooligosaccharides (COS), produced from chitin, becomes the focus because its characteristics like easy solubility and high absorptivity are apparently better than polymer sugar. Currently, few COS reports focused on its application in radiosensitization of cancer cell [9,10]. This research aims to investigate the elevating sensibility of human lung cancer line HepG2 treated with COS to radiotherapy, and to verify the auxiliary synergism of COS on radiotherapy in order to explore the therapy for cancer with better efficacy and lower damage.

## 2. Materials and methods

## 2.1. Cell line and experimental materials

Human lung cancer line HepG2 was purchased from Institute of Cell Biology, Chinese Academy of Sciences. Electron linear accelerator was purchased from Nanjing Chuang Rui Ying

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Biotechnology Co., LTD. CO<sub>2</sub> incubator was purchased from Shanghai GemTop Scientific Instrument Co., LTD. COS was purchased from Shanghai Hui Cheng Biological Technology Co., LTD. CCK-8 kit was purchased from Shanghai Li Rui Biotechnology Co., LTD.

## 2.2. Experimental methods

Cell lines HepG2 of seven concentration levels were created and HepG2 of each concentration was divided into 3 wells at the same time to make parallel samples. The cell lines HepG2 were cultured until the logarithmic phase. After digestion and dilution, the concentration was made into  $4 \times 10^4$ /mL and according to 0.1 mL/well, HepG2 was put into 96-well board for attachment culture in suitable environment. Twenty four hours later, the fresh culture medium of 0.11 mL/well diluted with COS was replaced and COS was added into each well with quantity of 0, 0.055, 0.11, 0.22, 0.33, 0.44, 0.55 mg. Twenty four hours after COS infiltration, CCK-8 reagent was added along the wall at 0.01 mL/well. A little tapping was for the good mixture of reagent and culture solution. Four hours after the full reaction, optical density (OD) in each group was determined at the wave length of 450 nm. The procedure was repeated 3 times to investigate the anti-proliferation effect of COS on HepG2 and hereby COS with concentration of 1.0 mg/mL was taken for the further research.

X ray of six exposure dose levels was created with RAY group and RAY + COS group in each dose level and each group was divided into 3 wells at the same time to make parallel samples. According to 200/well for exposure dose of 0, 1, 2 Gy, 400/well for 4, 6 Gy and 800/well for 8 Gy, X ray was inoculated on 6-well culture plate and single-cell suspension of different concentrations was added to it for attachment culture in the incubator in suitable condition. Six hours later, COS of certain quantity was added to each well in RAY + COS group to make the concentration of 1.0 mg/mL. Culture medium of the equivalent quantity was added to each well in RAY group for 24 h infiltration. The tissue analog of about 1 cm was capped on the culture plates in both groups and exposed to 6MvX ray, at the distance of 100 cm and dose rate of 2 Gy/min. The culture was continued for 10 d. After rinsing, fastening and staining, the number of cell cluster combined of more than 50 units was counted. The procedure was repeated 3 times to collect data and draw up the cell survival curve so that the sensitization enhancement ratio can be calculated by formula sensitization enhancement ratio =  $D_{0(RAY)}/D_{0(RAY + COS)}$ , where  $D_0$  was the final slope.

Three groups were created and each group was divided into 3 wells to make parallel samples. Cell suspension of  $1 \times 10^5$ /mL was inoculated into 6-well culture plate for attachment culture in the suitable environment for 24 h. Certain COS was added to RAY + COS group to make the concentration of 1.0 mg/mL. Culture medium of equivalent quantity was added to control group and RAY group. RAY group and RAY + COS group were exposed to 4 Gy X ray after 24 h infiltration. The culture was continued for 24 h after changing the fresh culture medium. After digestion, rinsing, dilution and other operation, the cell cycle and apoptosis rate were determined.

## 2.3. Statistical methods

SPSS17.0 software was used for statistical analysis. Measurement data were expressed by mean value  $\pm$  standard

deviation (mean  $\pm$  SD) and *t* test was applied. If  $P < 0.05$ , the differences were considered statistically significant.

## 3. Results

### 3.1. Anti-proliferation effect of COS on HepG2

Compared with the OD value without addition of COS, the other OD values were decreased with the increasing COS concentrations and the differences were statistically significant ( $P < 0.01$ ), which suggested that the amount of living cells was decreased with increasing COS quantity, the proliferation of HepG2 infiltrated with COS was inhibited and that the inhibition ratio and COS concentration was positively correlated. Detailed information was in Table 1.

### 3.2. Comparison of cell survival rates

Compared with the exposure dose of 0, the survival rates in RAY group and RAY + COS group were gradually decreased with increasing exposure dose and the decreasing range in RAY + COS group was bigger. The survival rates in RAY + COS group were slightly lower than in RAY group at the exposure dose of 1, 2 Gy but the differences were not statistically significant ( $P > 0.05$ ). When at the exposure dose of 4, 6, and 8 Gy, the survival rates in information were lower than in RAY group and the differences were statistically significant ( $P < 0.05$ ). Detailed information was in Table 2. The sensitization enhancement ratio of COS to HepG2 was 1.19.

### 3.3. Comparison of cell cycle distributions

Compared with control group, the percentages of cells at S phase in RAY group and RAY + COS group were significantly lower while cells at G<sub>2</sub>/M phase were higher; and both differences were statistically significant ( $P < 0.01$ ). Cells at G<sub>0</sub>/G<sub>1</sub> phase in RAY + COS group were relatively more and the difference was statistically significant ( $P < 0.05$ ). In contrast with RAY group, cells at S phase in RAY + COS group took up smaller percentage and cells at G<sub>2</sub>/M phase higher percentage, with statistically significant differences ( $P < 0.01$ ). The differences in duration ratio at G<sub>0</sub>/G<sub>1</sub> phase in both groups were not statistically significant ( $P > 0.05$ ). Detailed information was in Table 3.

The apoptosis rates in RAY group and RAY + COS group were much higher than in control group and the differences were statistically significant ( $P < 0.01$ ). The apoptosis rate in

**Table 1**

Anti-proliferation effect of COS on HepG2.

COS quantity (mg)	COS concentration (mg/mL)	OD (mean $\pm$ SD)	Inhibition ratio (%)
0.000	0.0	1.024 $\pm$ 0.005	0.00
0.055	0.5	0.938 $\pm$ 0.006*	8.35
0.110	1.0	0.841 $\pm$ 0.006*	17.90
0.220	2.0	0.754 $\pm$ 0.008*	26.37
0.330	3.0	0.650 $\pm$ 0.005*	36.51
0.440	4.0	0.584 $\pm$ 0.007*	44.72
0.550	5.0	0.458 $\pm$ 0.006*	55.19

\* $P = 0.000$  in comparison with COS of 0 mg.

**Table 2**

Comparison of cell survival rates in groups at different exposure doses.

Exposure rate (Gy)	Cell survival rate (%)	
	RAY	RAY + COS
0	99.17 ± 4.82	99.05 ± 3.23
1	92.73 ± 4.08	91.66 ± 3.72
2	74.87 ± 3.59	70.26 ± 2.35
4	42.33 ± 2.84	35.07 ± 3.32*
6	23.01 ± 2.77	16.82 ± 1.86*
8	5.64 ± 1.03	3.11 ± 1.15*

\* $P < 0.05$  in comparison with RAY group.**Table 3**

Comparison of cell cycle distribution.

Groups	S (%)	G <sub>0</sub> /G <sub>1</sub> (%)	G <sub>2</sub> /M (%)
Control group	48.33 ± 1.88	46.02 ± 2.83	5.65 ± 1.82
RAY group	35.27 ± 1.49*	50.50 ± 3.07	14.23 ± 1.36*
RAY + COS group	22.95 ± 1.54* $\Delta$	54.24 ± 2.76*	22.81 ± 1.56* $\Delta$

\* $P < 0.05$  in comparison with control group;  $\Delta P < 0.01$  in comparison with RAY group.**Table 4**

Comparison of cell apoptosis rate.

Groups	Cell apoptosis rate (%)
Control group	2.17 ± 1.24
RAY group	17.47 ± 1.83*
RAY + COS group	24.81 ± 1.62*

\* $P = 0.000$  in comparison with control group.

RAY + COS group was higher than in RAY group, with statistically significant differences ( $P < 0.05$ ). Detailed information was in Table 4 and Figure 1.

## 4. Discussion

Heavy haze raised lung cancer incidence and the close connection between PM<sub>2.5</sub> and lung cancer has been confirmed by researchers all over the world [11–13]. The annual growth rate

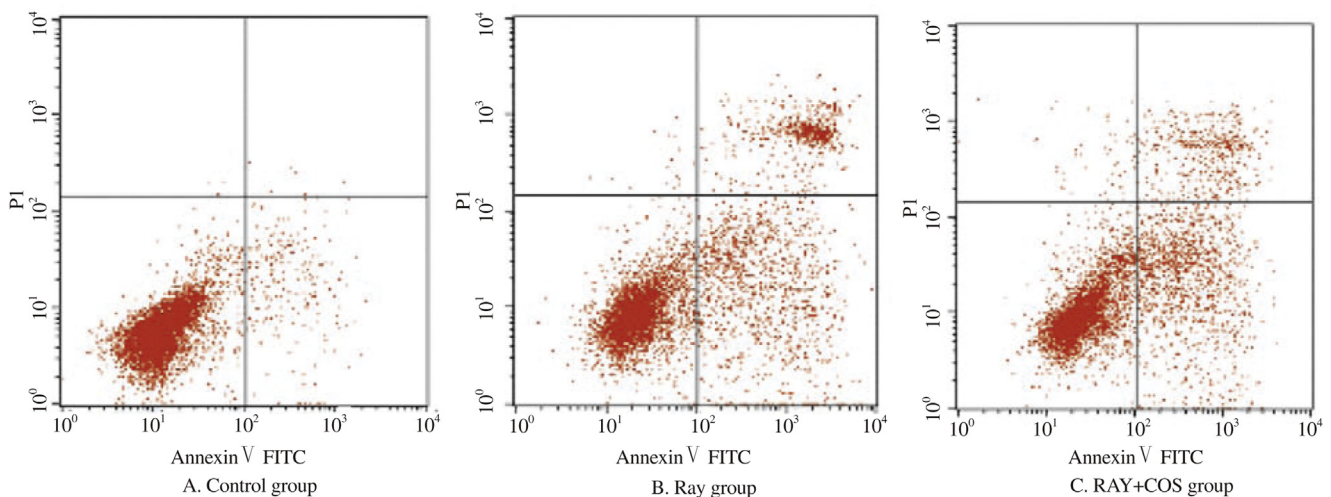
of lung cancer incidence in China is astoundingly over 25% and population that dies from lung cancer each year has increased to 4.5 times that of 1982 [14]. The present research aims to create a combined medication of COS and radiotherapy by taking the human lung cancer cell line HepG2 as the experimental object, to enhance the radiotherapeutic effect and reduce adverse effect so that to improve the life quality of cancer patients.

### 4.1. Direct anti-proliferation effect

The successful application of enzymolysis approach in the 1990s achieved the industrial production of COS [15]. That natural low molecular sugar polymer is compatible with human body and without adverse effect, has drawn the attention of medical investigators for its various physiological regulation functions [16–18]. The present research verifies that COS has the direct anti-proliferation effect on human lung cancer cell line HepG2 and the inhibitory rate is synchronously increased with the increasing COS quantity, which also verifies that COS expresses tumor-suppression activity against various cell lines. Kim and Karagozlu *et al* reported the inhibitory and killing effects of COS on leukemia HL-60 cell and gastric cancer AGS cell respectively [19,20]. Fernandes also established models to verify that COS inhibited the development of bladder tumor [21].

### 4.2. Promoting apoptosis of cancer cells

Ever since X ray and radium were discovered and applied to radiotherapy, radiotherapy has made great achievements in less than 100 years and become one of the major methods against cancer. Data revealed that the cure rate of cancers including oropharyngeal, tonsillar, maxillary cancer by radiotherapy was over 35% on average and early-stage cervical cancer even reached 86%–94%; radiotherapy was widely used before and after resection to shrink the tumor and eliminate the residue [22–24]. However, the collateral damage of radioactive rays could cause normal tissue, which is a huge burden and ordeal for patients. The present research testifies that RAY group and RAY + COS group could encourage mass apoptosis of cancer cells and RAY + COS group with stronger lethality is more efficient at the same exposure dose, which supports the theory that COS can regulate the natural apoptosis of cancer cells from multi-

**Figure 1.** Comparison of cell apoptosis.



aspects of gene and protein expression. Apoptosis is inevitable for normal cells, which is beneficial for the organism to keep alive and adapt to surroundings while cancer cells enter the abnormal growth model due to the loss of apoptosis regulation. Researches have demonstrated that COS can lower the expression of B-cell lymphoma 2 to open multiple pathways to stimulate apoptosis like reducing the intracellular glutathione level and animating oxidation, accelerating transmembrane transport of Ga<sup>2+</sup>, initiating inducing apoptosis of relevant inhibitor TG, activating cytochrome complex to shift from mitochondria to cytoplasm and producing superoxide anion [25-27]. In addition, COS can also elevate the Bax level, release Caspase and restrain synthesis of ATP to stimulate apoptosis [28].

#### 4.3. Changing cancer cell cycle distribution

The testing results of flow cytometry indicated that compared with control group, the duration at each stage of cell cycle in RAY group and RAY + COS group was changing. And RAY + COS group under the effect of COS had more significant change in contrast with RAY group; S phase was relatively shortened; G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases were relatively prolonged. Cell cycle kinetics provides theoretical guidance for radiotherapy; cells at M phase are the weakest with the lowest threshold value of being killed; cells at G<sub>2</sub> phase are the most seriously injured after exposure to rays with retarding speed of cell division; cells at S phase are the least affected by radiation [29]. The combined therapy of COS infiltration and radiotherapy can make cancer cells stay at G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phases to a larger extent and reduce the number of cells entering into S phase so that to improve the therapeutic effect of radiotherapy.

In conclusion, COS not only plays the direct anti-proliferation effect on HepG2 growth, but also promotes cells apoptosis, leads to the changing of proliferation process and efficiently improves the sensitivity of HepG2 to radioactive rays. When COS is combined with radiotherapy, it can greatly improve the therapeutic effect and meanwhile reduce the adverse damage on patients caused by radiotherapy. As the 'Jack of all trades' in medical field, COS possesses tremendous potential in clinical treatment.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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