

Basic Study

Chitooligosaccharides promote radiosensitivity in colon cancer line SW480

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Abstract

AIM: To investigate the anti-proliferation and radiosensitization effect of chitooligosaccharides (COS) on human colon cancer cell line SW480.

METHODS: SW480 cells were treated with 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL of COS for 48 h. CCK-8 assay was employed to obtain the cell survival ratio of SW480 cells, and the anti-proliferation curve was observed with the inhibition ratio of COS on SW480 cells. The RAY + COS group was treated with 1.0 mg/mL of COS for 48 h, while both the RAY and RAY+COS groups were exposed to X-ray at 0, 1, 2, 4, 6 and 8 Gy, respectively. Clonogenic assay was used to analyze cell viability in the two groups at 10 d after treatment, and a cell survival curve was used to analyze the sensitization ratio of COS. The RAY group was exposed to X-ray at 6 Gy, while the RAY+COS group was treated with 1.0 mg/mL of COS for 48 h in advance and exposed to X-ray at 6 Gy. Flow cytometry was employed to detect cell cycle and apoptosis rate in the non-treatment group, as well as in the RAY and RAY + COS groups after 24 h of treatment.

RESULTS: COS inhibited the proliferation of SW480 cells, and the inhibition rate positively correlated with the concentration of COS ($P < 0.01$). Cell viability decreased as radiation dose increased in the RAY and RAY+COS groups ($P < 0.01$). Cell viabilities in the RAY+COS group were lower than in the RAY group at all doses of X-ray exposure ($P < 0.01$), and the sensitization ratio of COS on SW480 cells was 1.39. Compared with the non-treatment group, there was a significant increase in apoptosis rate in both the RAY

and RAY + COS groups; while the apoptosis rate in the RAY+COS group was significantly higher than in the RAY group ($P < 0.01$). In comparing these three groups, the percentage of G₂/M phase in both the RAY and RAY + COS groups significantly increased, and the percentage of the S phase and G₀/G₁ phase was downregulated. Furthermore, the percentage in the G₂/M phase was higher, and the percentage in the S phase and G₀/G₁ phase was lower in the RAY + COS group *vs* RAY group ($P < 0.01$).

CONCLUSION: COS can inhibit the proliferation of SW480 cells and enhance the radiosensitization of SW480 cells, inducing apoptosis and G₂/M phase arrest.

Key words: Chitooligosaccharides; Cancer of colon; Radiotherapy; Radiosensitization; Apoptosis; Cell cycle

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Core tip: In this study, the colorectal cancer cell line SW480 that is homologous with colon-rectum was used as the research tool. It was confirmed that chitooligosaccharides (COS) not only directly blocked SW480 cell proliferation, but also enhanced radiotherapy effects. Furthermore, COS induced a large amount of SW480 cell apoptosis, and induced a large number of cells to remain in the G₂/M phase with radiation-sensitive killing effect. Thus, the sensitivity of SW480 cells to radiation was effectively enhanced 1.39 times. This is beneficial for the therapeutic effect.

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INTRODUCTION

According to the latest Chinese cancer epidemic profile survey, the 2010 cancer morbidity and mortality rates in China were 235.23/100000 and 148.81/100000, respectively. Among these cancers, colorectal cancer incidence and mortality have shown an obvious predominantly evident upward trend again, causing this to be the focus of medical experts^[1-4]. In China, colorectal cancer has a prevalence of 16.14%, allowing a leap up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 colorectal cancer cases were added nationwide; this total accounts for 18.6% in the world^[5,6]. China has become a big focus at the forefront of colorectal cancer research, since efficient and low toxicity treatments are needed. Since the discovery of radiation, radiotherapy has lasted for centuries as the

main indispensable weapon against cancer that is active in clinic. Statistics have shown that more than 70% and 50% of cancer patients are in need of this kind of therapy in China and the United States, respectively^[7,8]. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. However, radiosensitizers then emerged as a necessity of the times. Chitooligosaccharides (COS) are products of chitin, having good solubility and a high absorption rate, making the ratio of the carbohydrate polymer more advantageous for biological applications; and they have become the new favorite of medical researchers^[9,10]. Although there have been many reports about COS anticancer effects^[11-14], there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radio sensitivity for colorectal cancer cell line; this is reported below. We expect to further explore this superior and low-damage anticancer therapies.

MATERIALS AND METHODS

Seven COS concentration levels were established and 3-hole samples were simultaneously cultured in parallel with each level SW480 cells were subcultured to the logarithmic phase (human colon cancer cells SW480; Shanghai Cell Institute of Chinese Academy of Sciences). After digestion, the cells were diluted to a concentration of 5×10^4 cells/mL according to the 0.1 mL/hole access in a 96-well plate. A suitable environment was set (CO₂ incubator, Shanghai Gemtop Scientific Instrument CO.,Ltd.) for adherent growth, and diluted COS (Chitooligosaccharides, Shanghai Huich Biotech Inc.) was replaced after 24 h with fresh medium at 0.11 mL/hole, and added into each well at COS concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL After 48 h of COS application, CCK-8 reagent (CCK-8 Kit, Shanghai Liruibio Technology Co., Ltd.) was added along the pore walls at 0.01 mL/hole. Then, the culture plate was tapped to mix reagent and culture medium. After four hours of sufficient reaction, OD absorbance at $\lambda = 450$ nm was detected at all levels. The experiment was repeated three times to investigate the inhibitory effect of COS on SW480 cell proliferation. Accordingly, 1.0 mg/mL of COS concentration was selected for follow-up studies.

Six X-ray dose levels were established and levels were divided into the RAY group and RAY+COS group; each group was simultaneously cultured in parallel with the 3-hole sample. According to 0-, 1- and 2-Gy dose levels at 200/well, 4- and 6-Gy dose levels at 400/well, and 8-Gy dose level at 800/well inoculation amounts, respectively, different concentrations of single-cell suspensions in 6-well culture plate were set in an incubator with a suitable environment for growth

Table 1 Inhibition of chitoooligosaccharides for the proliferation of SW480 cells

COS concentration (mg/mL)	OD (mean \pm SD)	Inhibition rate
0	1.019 \pm 0.007	-
0.5	0.969 \pm 0.005 ¹	4.91%
1.0	0.908 \pm 0.008 ²	10.89%
2.0	0.804 \pm 0.006 ³	21.10%
3.0	0.692 \pm 0.007 ⁴	32.09%
4.0	0.580 \pm 0.006 ⁵	43.08%
5.0	0.433 \pm 0.008 ⁶	57.51%
P	0	-
F	137.6	-

Compared with COS concentrations in the 0 mg/mL Group: ¹ $P = 0.000$, $q = 17.437$; ² $P = 0.000$, $q = 31.326$; ³ $P = 0.000$, $q = 80.047$; ⁴ $P = 0.000$, $q = 99.096$; ⁵ $P = 0.000$, $q = 142.849$; ⁶ $P = 0.000$, $q = 165.379$. COS: Chitoooligosaccharides.

adherence. After six hours, appropriate amounts of COS were added into each well to reach a 1.0 mg/mL concentration in the RAY + COS group, while equal amounts of infiltrating medium were added into each hole and cultured for 48 h in the RAY group. Both groups were stamped with 1-cm thick tissue analogs in the culture plates and X-ray irradiated (Electron linear accelerator, Nanjing Chuang Rui Ying Biotechnology Co., Ltd.) at a distance of 100 cm with a dose rate of 2 Gy/min. Incubation continued for 10 d, the cells were washed, fixed and stained again; then, the number of cells were counted as 50 or more units of cell clusters. The experiment was repeated three times for statistical data, and the cell survival curve was draw up from the final slope of the D_{01} value obtained by sensitizing ratio $SER = D_{01(RAY)}/D_{01(RAY + COS)}$.

The three groups were established simultaneously in parallel with the 3-hole samples. The concentration of 1×10^5 /mL cell suspension was inoculated into 6-well culture plate with a suitable growth-adherent environment for 24 h. In the RAY + COS group, the correct amount of COS was added to reach a 1.0 mg/mL concentration. In the non-treatment group and RAY group, equal amounts of medium were added. In the RAY group and RAY + COS group, infiltration was carried out for 48 h and samples were exposed to 6-Gy X-ray irradiation. After replacing with fresh medium, cells were cultured for 48 h. After digestion, rinsing, dilution and other treatments were performed to determine the cell cycle stage and extent of apoptosis in each group.

Statistical analysis

Using SPSS19.0 statistical software for statistical analysis, OD value, survival rate, apoptosis rate and cell cycle distribution ratios were presented as mean \pm SD. OD values for each COS concentration level and cell survival rates at X-ray dose levels were compared among multiple groups using ANOVA analysis and compared between the two groups using SNK-q test. For the RAY group and RAY + COS group, cell survival rates under different X-ray doses, cell cycle control

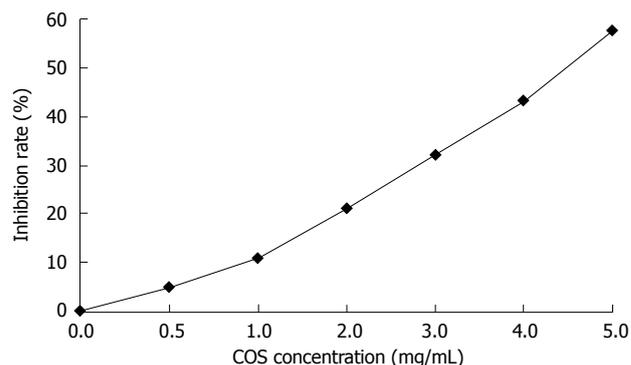


Figure 1 Inhibition curve of chitoooligosaccharides for proliferation of SW480 cells.

experiments among the three groups, and apoptosis rates between the three groups were compared by *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Inhibitory effect of COS on SW480 cell proliferation

In comparing the COS concentration of the OD value in the 0 mg/mL group, OD values progressively reduced at all levels as COS concentration increased, and the difference was statistically significant ($P < 0.01$). After 48 h of treatment with 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μ g/mL of COS concentration, SW480 cell survival rates were significantly lower than in the negative control group; and the differences were statistically significant ($P < 0.01$). The inhibition rate and the concentration of COS showed a positive correlation (Table 1, Figure 1).

Comparison of effects of different radiation dose on cell survival rate between groups

With an irradiation dose of 0 Gy as a reference standard, survival rate in the RAY and RAY + COS groups progressively reduced with increased radiation dose, and the difference was statistically significant ($P < 0.01$). The reduction in cell survival rate was greatest in the RAY + COS group. At dose levels 1, 2, 4, 6 and 8 Gy, survival rate in the RAY+COS group was significantly lower than in the RAY group, and the difference was statistically significant ($P < 0.01$). SER was 1.39 for COS in SW480 cells (Table 2, Figure 2).

Cell apoptosis rate between groups

Compared with proliferation in the non-treatment group, apoptosis rate in the RAY and RAY + COS groups increased sharply, the differences were statistically significant ($P < 0.01$). Apoptosis rate in the RAY+COS group also revealed a significant increase compared with the RAY group, and the difference was statistically significant ($P < 0.01$; Table 3, Figure 3).

Comparison of cell cycle distribution between groups

Compared with the non-treatment group, the proportion in G2/M phase in the RAY and RAY + COS

Table 2 Comparison of cells survival rates for different radiation doses between the RAY and RAY + COS group

Irradiation dose (Gy)	Cell survival rate (%)	
	RAY	RAY + COS
0	99.22 ± 3.51	99.17 ± 4.06 ¹
1	90.67 ± 3.82	85.30 ± 3.38 ²
2	73.69 ± 3.45	56.11 ± 2.95 ³
4	45.95 ± 3.41	28.64 ± 2.76 ⁴
6	23.84 ± 2.20	12.53 ± 2.03 ⁵
8	8.68 ± 1.75	3.81 ± 1.16 ⁶
P	0	0
F	182.7	243.2

Compared with RAY group: ¹P = 0.978, t = 0.028; ²P = 0.006, t = 3.158, ³P = 0.000, t = 11.619; ⁴P = 0.000, t = 11.837; ⁵P = 0.000, t = 11.335; ⁶P = 0.000, t = 6.959. COS: Chito oligosaccharides.

Table 3 Comparison of cell apoptosis rates among the three groups

Groups	Cell apoptosis rate
Non-treatment group	1.79 ± 0.37
RAY group	9.33 ± 1.05 ¹
RAY + COS group	22.64 ± 1.27 ^{2,3}

Compared with the control group: ¹P = 0.000, t = -20.318; ²P = 0.000, t = -47.286; compared with the RAY Group: ³P = 0.000, t = -24.232. COS: Chito oligosaccharides.

Table 4 Comparison of cell cycle distribution among the three groups

Groups	S (%)	G0/G1 (%)	G2/M (%)
Non-treatment group	30.15 ± 0.82	39.41 ± 1.05	30.44 ± 0.68
RAY group	22.48 ± 0.74 ¹	30.51 ± 0.86 ²	47.01 ± 0.52 ³
RAY + COS group	18.89 ± 0.65 ^{4,7}	24.34 ± 0.46 ^{5,8}	56.77 ± 0.28 ^{6,9}

Compared with the control group: ¹P = 0.000, t = 20.832; ²P = 0.000, t = 19.672; ³P = 0.000, t = -58.070; ⁴P = 0.000, t = 32.283; ⁵P = 0.000, t = 39.438; ⁶P = 0.000, t = -107.412; compared with the RAY group: ⁷P = 0.000, t = 10.935; ⁸P = 0.000, t = 18.979; ⁹P = 0.000, t = -49.577. COS: Chito oligosaccharides.

groups significantly increased, while the proportions in S and G0/G1 phase all reduced; the differences were statistically significant (P < 0.01). Compared with the RAY group, the proportions in S phase and G0/G1 phase in the RAY + COS group were smaller, while the G2/M phase was significantly longer; the differences were statistically significant (P < 0.01). See Table 4, Figure 4.

DISCUSSION

Rapid economic growth gives rise to the rapid development of science and technology. However, improvements in medical technology have failed in stopping cancer from affecting human health. Modern unhealthy diets and living habits stimulate and mainly cause the continuous rise in colorectal cancer

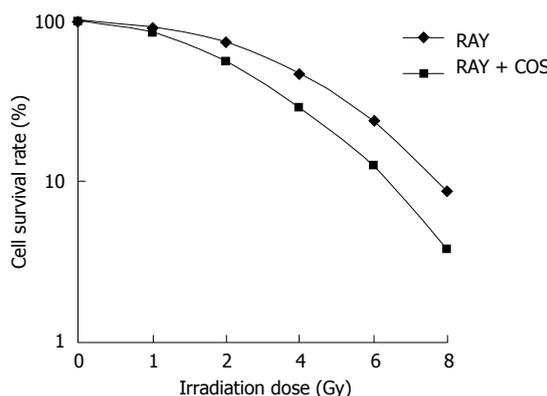


Figure 2 Cells survival rates at different radiation doses between the RAY and RAY + COS groups. COS: Chito oligosaccharides.

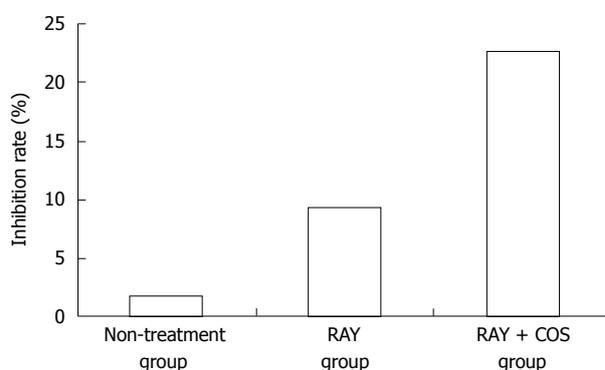


Figure 3 Cell apoptosis comparison between the three groups.

morbidity^[15-17]. Statistical data from international cancer research institutions have indicated that China's 2012 annual new-onset cases of colorectal cancer reached 1.47 times that of cases in 2006, and the total incidence grew by nearly 50% in six years^[18]. Regarding the incidence of colorectal cancer in China, the annual growth rate rose sharply to more than two times the international average^[19]. This situation is not optimistic, and the exploration of more effective drugs and treatment without delay is of great significance.

Inhibitory effect of COS on proliferation of colon cancer SW480 cells

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity^[20-22]. Particularly, it has anti-tumor effects, which have been a research focus for domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth-blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect^[23-25]. In this study, the colorectal homologous colorectal SW480 cell line was the target. This study confirms that COS directly

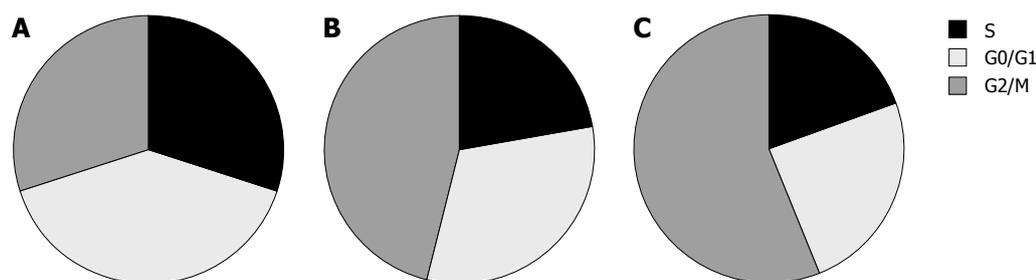


Figure 4 Comparison of cell cycle distribution among the three groups. A: Non-treatment group; B: RAY group; C: RAY + COS group. COS: Chitooligosaccharides.

blocks SW480 cell proliferation, and its inhibitory effect increases with the concentration of COS application showing increase in increments; 5 mg/mL of COS treatment for 48 h induced SW480 cell viability to decrease by 57.51%.

COS enhances SW480 cell sensitivity to radiation

Radiotherapy has an irreplaceable position in the treatment of cancer. It is widely used in various stages of the disease course; preoperatively it shrinks tumors to create conditions for radical enterectomy, it removes residues postoperatively to prevent recurrence, and it cooperates with chemotherapy to prevent metastasis. Early radiotherapy for nasopharyngeal carcinoma, skin cancer and cervical cancer has a possibility of more than 9% cure^[26-28]. Early exposure to radiotherapy also can improve the 5-year survival rate from 70% to 80% for esophagus and prostate cancer^[29,30]. Although radiotherapy has significantly prevents pain in patients with organ disease, there are also shortcomings. Radiation attack precision is limited, and often implicates normal tissue surrounding lesions, leading to tissue damage, which adds to the double burden of cancer patients both physically and mentally. This study found that X-rays have a mass destruction effect on SW480 cells, and cell death increased with radiation dose; while before radiotherapy, COS application obviously makes the cell survival rate decrease, COS can effectively increase SW480 cell sensitivity to radiation by 1.39 times. Furthermore, this controlled experiment has shown that the RAY + COS group had significantly improved treatment efficacy with the increase in apoptosis rate, and the cell cycle distribution changed significantly.

COS achieves radiosensitization by promoting SW480 cell apoptosis

Apoptosis maintains homeostasis in the body. However, it has an independent regulatory program that takes the initiative to open the "die" mode to conserve limited resources when cells are faced with adverse living conditions. Tumor cells lose this order of regulation and fall into a disordered and uncontrolled proliferation cycle. Radiation-induced apoptosis has been demonstrated for a long time^[31-34]. COS combined

with radiotherapy reverses the deactivation of the mechanism of apoptosis in a cancer cell to a greater degree, and pulls it back to its normal life trajectory. The complexity of the entire process of cell apoptosis involves cooperation of multiple genes and proteins. Krysko's research has indicated that COS can activate apoptosis promoter Caspase-3^[35,36]. Tan believes that COS can damage mitochondrial membrane stability and release Cyt C into the cytosol^[37]. Mates has also reported that COS can down-regulate environmental GSH activity and stimulate oxidative damage^[38,39]. A number of conclusions have confirmed that COS has a positive effect on the apoptosis of tumor cells.

COS achieves radiosensitization by changing the SW480 cell cycle distribution

The mechanism of action of radiotherapy is to destroy DNA strand integrity including breaking the connection of ester bond sequences and destroying base modifications. From the initial point of its life cycle, radiotherapy blocks various physiological functions of the tumor cell and genetic information delivery^[40,41]. Cell cycle distribution has a deep influence on radiotherapy^[40,42-45]. Flow cytometry analysis revealed that COS treatment leaves a large number of SW480 cells stranded in the G2/M phase that is very sensitive to radiation, while reducing the G1 phase and S phase that are responsible for DNA damage repair, reducing the resistance of cancer cells to radiotherapy and enhancing its therapeutic effect. Radiation biological research pointed out that in order to ensure a smooth and orderly replication, the whole proliferation process speed is controlled at G1, S and G2 levels, respectively, by three regulatory processes^[46-49], speculating that COS control in cell cycle distribution is most likely related to the start-up and expression of these three processes.

In summary, COS not only directly arrests SW480 cell growth, but also helps radiotherapy. COS induces SW480 cells apoptosis accompanied by a large number of proliferation process changes. Thus, this greatly enhances radiation lethality and has a beneficial therapeutic effect. In-depth exploration of COS as radiosensitizer is expected to bring a new dawn for the life of colorectal cancer patients.

COMMENTS

Background

Modern unhealthy diets and living habits are major causes that stimulate the prevalence of colorectal cancer to continue to soar. In China, colorectal cancer has a prevalence of 16.14%, which has clinically already leapt up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 cases of patients were added nationwide; this total accounts for 18.6% in the world. China has become a big focus in the forefront of colorectal cancer research, and efficient and low toxicity treatments are needed. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. Radiosensitizers have now emerged as a timely aid.

Research frontiers

In order to reduce the toxicity of radiotherapy, current research has focused on primarily two aspects: increase in the accuracy of positioning, and enhancement of radiation radiosensitivity of tumor tissues. Using radiation sensitizers has become popular because it is simple to apply. Previous studies have shown that radiosensitization mechanisms include improved cell hypoxia, increased DNA damage and influence of the cycle phase distribution. In addition to 5-fluorouracil, cisplatin and gemcitabine, the conventional radiotherapy sensitizers, C225, L-778-123 and COX-2 inhibitors and other new sensitizers have gained attention in recent years. Further interdisciplinary approaches have also started to introduce new drugs and new mechanisms of action in the field of radiation sensitizer agents.

Innovations and breakthroughs

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity. Particularly, it has anti-tumor effects, which has been a research focus by domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect. Although there are many reports about COS anticancer effects, there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radiosensitivity in colorectal cancer cell lines.

Applications

This study showed that chitooligosaccharides can effectively enhance the sensitivity of SW480 cancer cells to radiation; chitosan oligosaccharide combined with radiotherapy treatment would be helpful and promising for colorectal cancer. Regarding the effect of chitooligosaccharides on radiosensitization, an in-depth study would be expected to explore this efficient and low-damage anticancer therapeutic breakthrough.

Peer-review

This is a very interesting study about the chitooligosaccharide promotion of radiosensitivity in a colon cancer line. The study is well designed and the manuscript is well written.

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