Original Article

Combined Effect of Sodium Selenite and Ginsenoside Rh2 on HCT116 Human Colorectal Carcinoma Cells

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Abstract

Background: Sodium selenite and ginsenoside Rh2 (G-Rh2) are well known for their anticancer properties and have been exploited as a new therapeutic approach. In this study, we are interested to evaluate if sodium selenite and G-Rh2 combination results in a synergistic anticancer effect that could contribute to lower systemic toxicity.

Methods: We observed the synergistic antitumor effect by combination of sodium selenite and G-Rh2 on HCT-116 human colorectal carcinoma cells in vitro. Cell growth, viability, cell cycle progression and cell apoptosis, Bax/Bcl2 ratio, caspase-3 expression, reactive oxygen species (ROS) production and autophagy were evaluated.

Results: The results showed that sodium selenite and G-Rh2 combination have a synergistic effect on cell growth inhibition (57%) compared with sodium selenite (25%) and G-Rh2 alone (28%) after 24 hours of treatment. This combination also induced G1 and S phase arrest simultaneously and increased apoptosis rate. The results also indicated that Bax/Bcl2 ratio and caspase-3 expression, known as proapoptotic factors, were increased in the presence of sodium selenite and G-Rh2 alone. However, combined drug treatment results in a more significant increase in Bax/Bcl2 ratio and caspase-3 expression ($P < 0.05$). In addition, this combination significantly induces a depletion of ROS production and autophagy, compared to control, sodium selenite and G-Rh2 alone ($P < 0.05$).

Conclusion: Sodium selenite and ginsenoside Rh2 combination may be a more effective treatment for human colorectal carcinoma and is a promising chemotherapeutic approach for malignant tumors.

Keywords: Colorectal carcinoma, ginsenoside-Rh2, sodium selenite, synergistic effect

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Introduction

Colorectal cancer is one of the most common cancers and a major cause of cancer-related deaths worldwide, and it runs after gastric cancer, lung cancer and esophageal cancer in China.[1] It will become one of the most malignant tumors with high incidence, increasing morbidity and mortality. Despite the primary treatment for colorectal cancer, including surgical resection and chemotherapy (most often by 5-Fluorouracil), it is an important for clinicians and researchers to treat colorectal cancer, using more effective chemotherapy drugs and novel approaches.

Selenium, an essential trace element for human health, has attracted great interest in cancer chemoprevention. And the mechanism for its non-specific tumor inhibitive effect is combination of various functions such as the prevention of DNA damage, oxidative stress and inflammation.[2,3] Selenium compounds with higher concentrations have antitumor activity by inducing cell growth inhibition and apoptosis on cancer cells in vitro concomitantly with an increase in Bax/Bcl2 ratio, Bak and Bid proteins, as well as decrease in Bcl2 expression.[4–6] Nadiminity, et al.[7] reported that selenium may also activate enzyme detoxification mechanisms by selenoproteins activation as glutathione peroxidase (Gl-Ppx). Sodium selenite, a major inorganic form of selenium, is most often used in chemo-preventive studies.[8] Nevertheless, the anti-tumor effects of selenium occur in a limited range of dosages, below which it cannot perform its anticancer effects and above which it shows the toxicity to the human health. Therefore, it is essential to seek another kind of synergistic antitumor drug with low side effect, which can enhance the tumor killing effect of sodium selenite.

On the other hand, a large number of natural plants are widely used as functional health products for additional nutritional supplements, which can provide many desirable health benefits beyond basic nutrition and prevent chronic diseases.[9] Ginseng, one of the most well-known botanical supplements, originates in oriental countries. Previous studies confirmed that ginsenosides are the main active ingredients in ginseng, and has a wide variety of biological activities including: immunity improvement, anti-inflammatory and anti-tumor effect.[10] Ginsenoside-Rh2 (G-Rh2) has been isolated from red ginseng and shown to have beneficial impacts on tumor prevention.[11,12] Interestingly, Jia, et al. reported that G-Rh2 has marvelous synergy effects of some chemotherapeutic drugs, even at a non-effective dosage.[13,14] This Biological characteristic provides a basis for finding a drug combination of cancer treatments.

Due to the lack of curative treatments for the advanced colorectal cancer, it is important to explore additional trials for the successful development of an effective therapeutic approach. In this study, we aim to evaluate if sodium selenite and G-Rh2 combination results in a synergistic anticancer effect that could contribute to lower systemic toxicity.

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Materials and Methods

Cell culture

Human colorectal carcinoma cell line, HCT-116, was purchased from the American Type Culture Collection (ATCC). Cell growth was performed in RPMI 1640 medium (Sigma) with 10% (v/v) fetal bovine serum (FBS, Biochrom), and 2 mM L-glutamine (Sigma), supplemented with 100 U/mL Penicillin, 100 µg/mL Streptomycin. Cells were cultured in a 95% humidified incubator with 5% CO_2 at 37ºC, and passaged with 0.25% trypsinization every fourth day. For cell detection, HCT-116 cells were seeded in a concentration of 4 × 105 cells/mL. After being cultured up to 80% confluence, cells were washed twice with fresh assay medium and treated for 24~72 h with sodium selenite (Sigma) (10 nM to 10 µM) and/or G-Rh2 (Ronghe biotech. Com., shanghai) (0.5 µM to 50 µM) dissolved in 15% (v/v) ethanol.

Cell growth inhibition assays

Cell proliferation was measured by the cell counting kit-8 (CCK-8, Dojiondo) method.[15] Absorbance values were normalized to the values obtained from the control cells. Briefly, after the treatment on a 96-well plate, each well of the plate was added to 10 µL of CCK-8 solution. Cells were then incubated at 37ºC for 2 h, and the absorbance value at 450 nm were measured in a microplate reader (BioRad, Model 680). Half maximal inhibitory concentration were abbreviated as "IC50".

Cell cycle analyses

Cell cycle distribution was evaluated at 0, 24, and 72 hours, respectively by analyses of DNA content according to the previous reports.[16] Briefly, 1 × 10^6 cells were collected and treated with 1 mL of pre-cold propidium iodide (PI, sigma) solution (0.5 mg/mL RNase A, 50µg/mL PI in PBS). Samples were then incubated at room temperature in the dark for 10 min and immediately analyzed by flow cytometry (BD FACScalibur, BD Bioscience).

Cell apoptosis assay

The apoptotic rate of cultured HCT-116 cells was detected with FCM analysis. Apoptotic cells were labeled by Annexin V-FITC/PI double staining. Briefly, the samples were washed twice and adjusted to a concentration of 1 × 106 cells/mL with pre-cold PBS. To each 100 µL of cell suspension, 5 µL of Annexin V-FITC and 5 µL of PI were added. Then, samples were incubated for 15 min at room temperature in the dark. Finally, the apoptotic rate of cells was determined at 488 nm under FCM.

Apoptosis related molecules assays

Determination of Bax, Bcl2 and caspase-3 were conducted by flow cytometry after incubating the cells with monoclonal antibodies labeled with fluorescent probes (Santa Cruz Biotechnology). Cells were permeabilized and fixed with 250 µL of cytofix–cytoperm (Cytofix/cytoperm kit, Pharmigen) for 20 min at 4ºC and washed with perm-wash (Cytofix/cytoperm kit), followed by centrifugation at 1000 rpm for 5 min.

Cell samples were labeled with 2 µg phycoerythrin combined Bax antibody and 1 µg fluorescein isothiocyanate (FITC) conjoint Bcl2 antibody respectively. Samples were then followed by incubation for 15 min in the dark and rinse with phosphate buffer (pH = 7.4) one time. For caspase3 detection, cells were labeled with 2 µg caspase 3 monoclonal antibody combined with phycoerythrin, followed by incubation in the dark for 30 min.

Reactive oxygen species determination

Intracellular levels of peroxides were quantified according to the previous study.[17] Briefly, 5 × 10^5 cells were incubated with 5 uM 2,7-dichlorofluorescein diacetate (DCFH2-DA) (Sigma) at 37ºC for 60 min in the dark, washed twice with phosphate-buffered saline (PBS), resuspended in 0.5 mL PBS and immediately analyzed by flow cytometry. This assay is based on the conversion of (DCFH2-DA) to DCFH2 by intracellular esterase. Upon excitation at 488 nm, the intensity of green fluorescence is proportional to the intracellular level of ROS.

Transmission electron microscopy (TEM)

After incubation with sodium selenite and/or G-Rh2 for 24 hours, the cells were harvested by trypsinization, washed twice with PBS, fixed with pre-cold 4% glutaraldehyde for 2~4 h and post-fixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections (70 nm) were cut, and stained with 0.2% lead citrate and 1% uranyl acetate. Images were observed and captured under a TEM (Jeol, JEM-1230) at 80 kV.

Statistical analyses

All the data are expressed as mean ± SD. Significance was assessed for P-values < 0.05 according to t-tests.

Results

Antiproliferative effect of sodium selenite and G-Rh2 on colon cancer cells

Sodium selenite and G-Rh2 inhibit colon cancer cells (HCT-116) growth in a dose and time dependent-manner. Half maximal inhibitory concentration values (IC50) of sodium selenite (Figure 1A) and G-Rh2 (Figure 1B) were reached for approximately 10 µM and 50 µM respectively, after 24 h incubation. However, when cells were treated with 2.5 µM sodium selenite and 12.5 µM G-Rh2 simultaneously, there was a synergistic antiproliferative effect (Figure 1C). It suggested that sodium selenite and Rh2 combination have a synergistic effect on cell growth inhibition.

Cell cycle arrest

Using flow cytometry, cell cycle distribution of untreated cells, sodium selenite and/or G-Rh2 treated cells were measured to determine if the suppressive effect has been caused by specific perturbing cell cycle-related events.

The results showed that sodium selenite treatment blocked the cell cycle at the S boundary, and approximately 46% cells were accumulated at this phase. After G-Rh2 treatment, there showed a G1 arrest, and there about 53% cells were blocked in G1 stage. However, the combination of drugs induced G1 and S phase arrest simultaneously, reflecting a synergistic effect (Table 1).

Cell apoptosis rate analysis

To investigate if sodium selenite and G-Rh2 combination has the synergistic effect of inducing the HCT-116 cell apoptosis, the apoptotic rate was quantitatively analyzed by FCM. As shown in Figure 2, sodium selenite (2.5 µM) and G-Rh2 (12.5 µM) could increase the apoptotic rate in HCT-116 cells compared to the

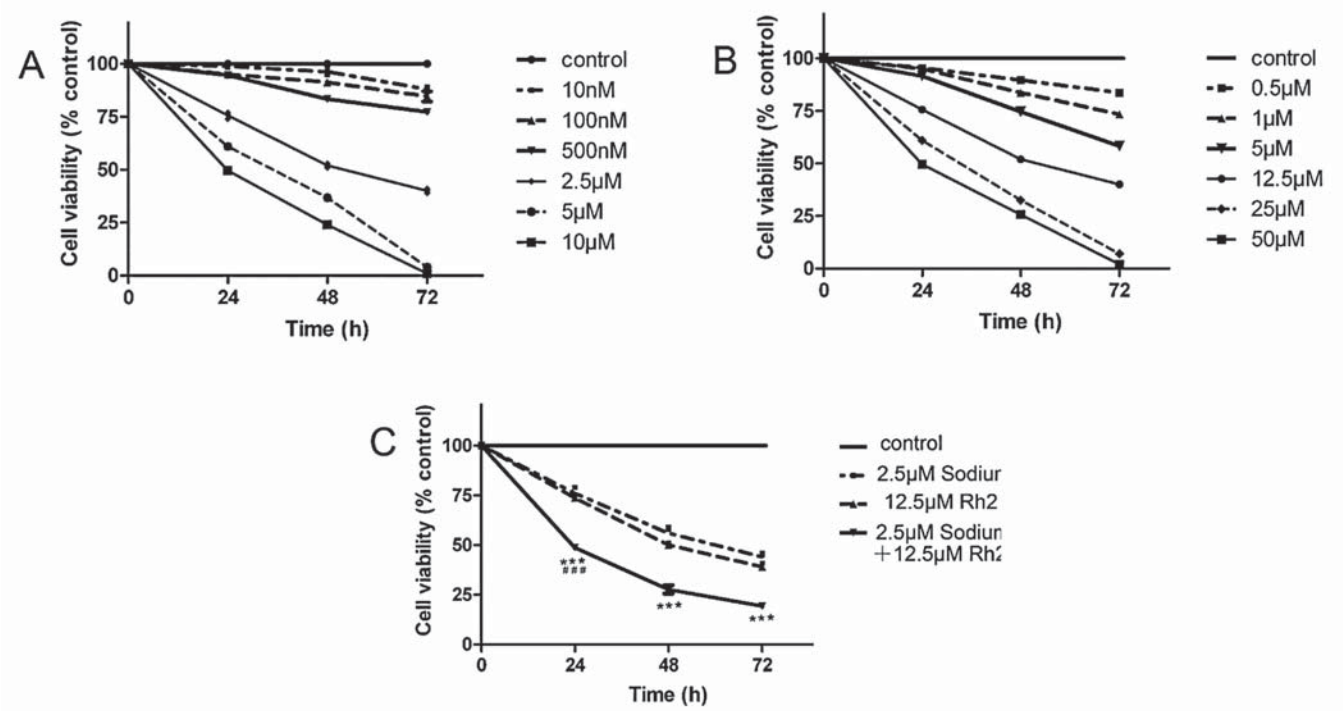

Figure1. Dose–response curves. Effect of sodium selenite **A)** G-Rh2; **B)** alone and in combination; **C)** on HCT116 cells growth was evaluated. Results are expressed as mean ± SD. Significant differences of combined effect relative to controls, namely, 2.5 μM sodium selenite and 12.5 μM G-Rh2 are considered for ***$P < 0.001$ and ### $P < 0.001$, respectively.

Table1. Effect of sodium selenite, G-Rh2 and in combination on HCT116 cell cycle after 0, 24 and 72 hours treatment, respectively (mean ± SD, %).

Cell cycles	G1			G2M			S		
	0	24	72	0	24	72	0	24	72
Control	71.1 ± 1.3	32.3 ± 0.9	49.7 ± 2.0	15.2 ± 1.1	30.9 ± 2.1	22.8 ± 0.4	13.7 ± 0.7	36.8 ± 1.7	27.5 ± 0.2
2.5 μM sodium selenite*s	24.4 ± 0.3	23.1 ± 0.2	21.7 ± 0.5	29.3 ± 2.1	19.5 ± 0.6	33.1 ± 2.0	46.3 ± 1.0	47.4 ± 2.3	45.2 ± 1.9
12.5 μM G-Rh2*s	56.3 ± 1.7	52.2 ± 2.0	50.1 ± 0.9	25.1 ± 0.1	22.5 ± 2.0	19.5 ± 2.3	18.6 ± 0.5	25.3 ± 1.0	30.4 ± 3.0
2.5 μM sodium selenite+12.5 μM G-Rh2*s	41.1 ± 0.9	45.6 ± 2.3	42.7 ± 1.4	15.7 ± 0.1	12.1 ± 0.2	18.3 ± 1.1	43.2 ± 1.7	42.3 ± 1.9	39.0 ± 2.6
*Significant alterations compared to control.									

control group, and the apoptotic rates were approximately 7.6%, 13.0% and 1.9%, respectively, after 24 h of treatment. While the apoptotic rate in combined group was about 28.0%, which was significantly higher than the apoptotic rate in the control group.

Expression of apoptosis- related molecules

Apoptosis- related molecules Bcl2 and Bax are members of the Bcl2 family, play an anti-apoptotic and proapoptotic role, respectively. Caspase-3 expression was evaluated, to analyze some of the mechanisms, which may participate in cell death induced by sodium selenite alone and in combination with Rh2, Bax/Bcl2 ratio. Figure 3 showed an increase in the apoptotic molecules caspase-3 expression and Bax/Bcl2 ratio in all drugs treatment groups, compared to control. However, there was significant difference in caspase-3 expression and Bax/Bcl2 ratio between the drugs of combination group and one drug-induced group.

ROS production

The ROS levels in HCT-116 cells were evaluated to examine, whether sodium selenite and G-Rh2 combination-induced cytotoxicity could be associated with ROS production. As shown in Figure 4, this combination induced ROS depletion in HCT-116 cells compared to sodium selenite and G-Rh2alone, after 24 h treatment.

Transmission electron microscope

The ultra-structural information of sodium selenite and Rh2 treated cells was examined using a TEM. After 48 h treatment of two kinds of inducers, cells were collected and analyzed. Untreated cells showed no obvious autophagic features (Figure 5A). In sodium selenite or Rh2 treatment group, there were very few numbers of cytoplasmic inclusions that were wrapped in a double-membrane in the cytoplasm were observed (Figures 5B and 5C). However, there were numerous autophagosomes in the cytoplasm of HCT-116 cells, exposing on combination of sodium selenite and G- Rh2 (Figure 5D).

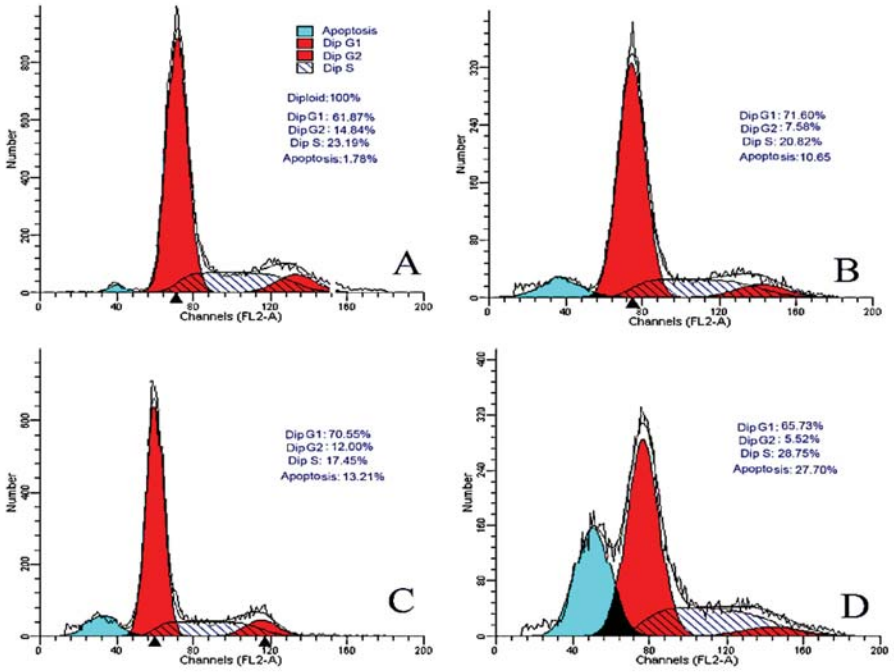

Figure 2. FCM analysis of cell apoptosis rate after treatment with differential inducers for 24 h. **A)** Control; **B)** 2.5 μM Sodium selenite; **C)** 12.5 μM G-Rh2; **D)** In combination.

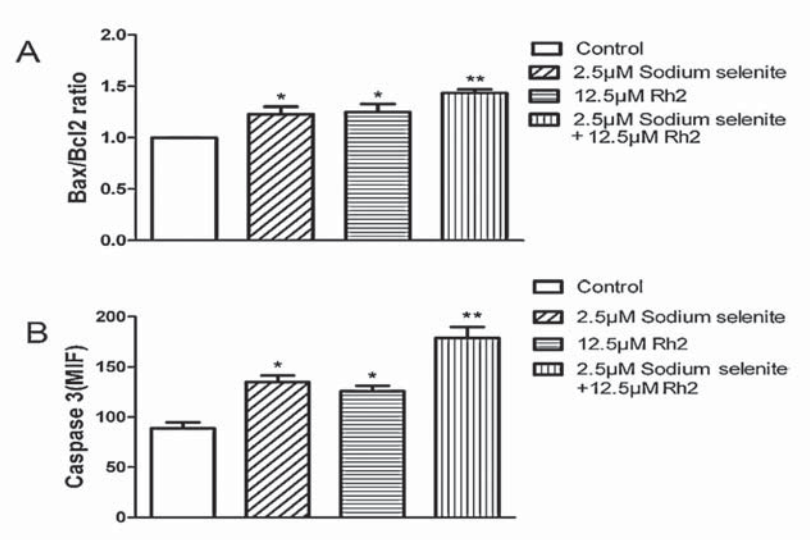

Figure 3. Effect of sodium selenite and G-Rh2 on Bax/Bcl2 ratio **A)** and caspase-3; **B)** following 24 h treatment were evaluated on HCT116 cells. The data are expressed as mean ± SD. *P < 0.05 vs. control.

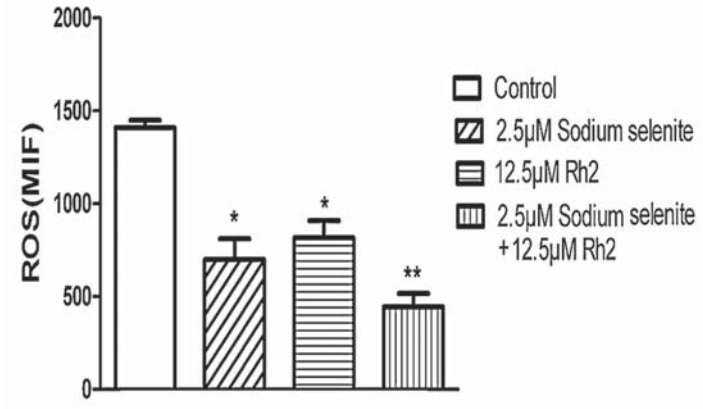

Figure 4. Evaluation of ROS levels in HCT116 cells treated with sodium selenite, G-Rh2, and in combination. The results were expressed as the means intensity fluorescence (MIF) ± SD. Significant differences of ROS production relative to control, sodium selenite and G-Rh2 are considered for **P < 0.001 and *P < 0.05, respectively.

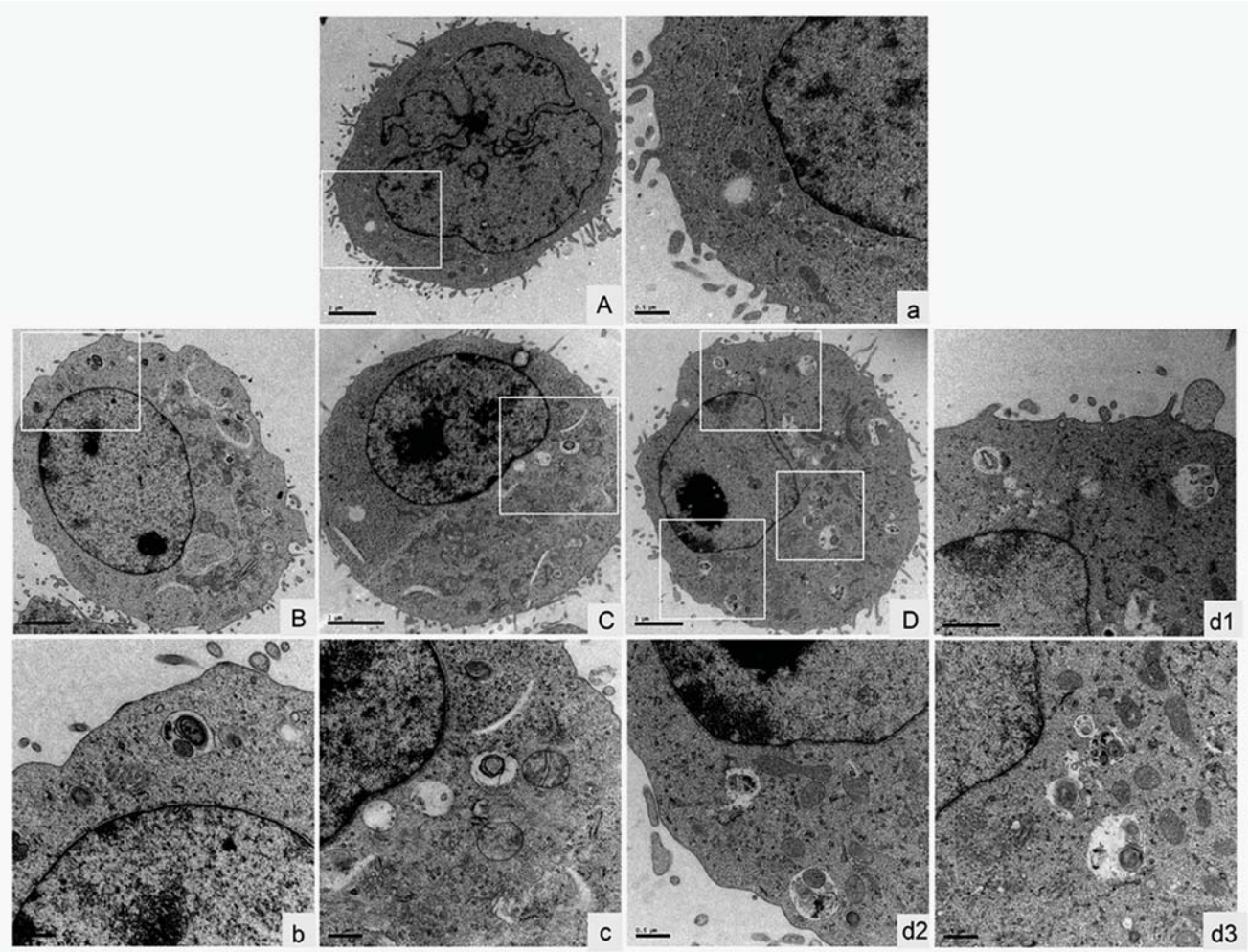

Figure 5. Transmission electron microscopy images of autophage. **A)** Cells were control; **B)** and treated with 2.5 μM sodium selenite; **C)** 12.5 μM G-Rh2; **D)** and in combination respectively, for 24 hours; **a, b, c, d (d1, d2, d3)** are higher magnifications of boxed areas (in A, B, C and D).Scale bars, as indicated in the images.

Discussion

In recent decades, diagnosis and treatments for cancers have improved greatly. However, the incidence of colorectal cancer is increasing worldwide. Due to difficulties in early diagnosis and treatments, colorectal cancer has been associated with poor prognosis. Large clinical trials have demonstrated that an elevated selenium intake may notably be associated with reduced cancer risk or the development, dissemination and recidivating of cancer.[18–20] Mariana, et al. demonstrated that sodium selenite induced PC3 metastatic prostate cancer cells death in a dose and time dependent manner.[16] Also, they found that sodium selenite has a synergistic antitumor effect by combining with docetaxel. On the other hand, ginsenoside Rh2, one of the main components of ginseng, has also attracted much research interesting for its anticancer characteristics.

Therefore, we are interested to evaluate if sodium selenite and ginsenoside Rh2 combination results in a synergistic anticancer effect on colorectal cancer cells that could also contribute to systemic toxicity reduction.

Our results indicated that sodium selenite and G-Rh2 combination play synergistic anti-proliferative and cytotoxic effects on HCT-116 cells. This synergistic anti-tumor role can be achieved by using a lower dose of drug exposure. Such a characteristic of synergistic effect can contribute to the systemic toxicity reduction, and it is a very important aspect in clinical application. As documented previously, sodium selenite and Rh2 alone inhibits cell growth, by arresting cells to the growth phase, including G2/M, G1/S and S respectively.[4,21,22] In the present study, we also found that combination of sodium selenite and G-Rh2 strongly inhibits the growth of HCT-116 cells, and induces Gl phase-arrest and S phase-prolongation.

In the process of programmed cell death that triggered by following exposure of cells to stimuli, the protein Bcl-2 interferes with the activation of caspases by preventing the release of cytochrome c, while this release can be induced by Bax.[23] Thierry et al.[24] demonstrated that Bcl-2 prolongs cell survival after Bax-induced release of cytochrome c, thus inhibits the programmed cell death (apoptosis). Therefore, it is helpful to understand the effect on apoptosis induction by sodium selenite, ginsenoside Rh2, or in combination through the evaluation of Bax/Bcl2 ratio. Previous researches had proved that sodium selenite induces an increase in Bax/Bcl2 ratio and caspase-3 expression.[16,25] Kim, et al. demonstrated that Rh2 induces apoptosis via activation of caspase-1 and -3 and up-regulation of Bax in human neuroblastoma.[26] Our results are also indicated that both sodium selenite and G-Rh2 could promote an increase in Bax/Bcl2 ratio and caspase-3 expression, which were significantly augmented by two drugs of combina-

tion. However, it has reported that G-Rh2 can induce apoptosis independently of Bcl-2, Bcl-xL, or Bax pathways in rat glioma C6Bu-1 cell line,[27] suggesting that G-Rh2 can induce cell apoptosis through multiple pathways. Cell apoptosis rate analysis results in this study confirmed that sodium selenite and G-Rh2 in combination can greatly enhance the apoptotic rate of HCT-116 cells. However, we cannot exclude other apoptotic pathways or proteins that may be involved the cell death process, such as membrane or extrinsic apoptotic pathways.[16]

ROS plays the role of either inducing cell death or activating some survival pathways to protect cells from death. The exact effect of ROS on a particular cell type is likely depending on the nature and levels of ROS that be induced. Moreover, it could be a result of peroxides conversion into hydroxyl radicals. Previous reports have shown that both sodium selenite and Rh2 can induce ROS secretion in some cell lines.[16,28,29] We also surprisingly found that sodium selenite and Rh2 combination can greatly induce ROS depletion after 24 h of treatment. It suggested that this combination may more effectively inhibit the antioxidant system, and then leads to oxidative lesion by reactive hydroxyl radicals and consequent cell death.

Autophagy is a highly conserved survival response to growth limiting conditions, such as nutrient depletion, hypoxia and the presence of cytotoxic drugs,[30,31] and involve in many physiological and pathological processes, including infections, neurodegeneration, myopathies and cancers.[32] The role and mechanism of autophagy in carcinogenesis is still uncertain to date, but it is reported that activation of autophagy may block tumor growth in the early stage of carcinogenesis.[33] Transmission electron microscopy technique is one of the most widely used methods for morphological observation of autophagosome. Multiple studies have shown that sodium selenite and Rh2 alone can also induce autophagy in vitro in various cancer cells, including leukemia HL60 cells,[34] human lung carcinoma cells[35] and human colorectal cancer cells.[36] In this study, sodium selenite (2.5 μM) and G-Rh2 (12.5 μM) combination induces the accumulation of giant autophagosomes, which were double membrane-enclosed vesicles containing organelles in cells. TEM results suggest the synergetic anti-cancer cells effect of the combination drug, to a certain extent, is dependent on the activation of autophagic cell death.[16]

In conclusion, our results showed that sodium selenite and G-Rh2 combination plays a synergistic colorectal cancer cells killing activities through multiple mechanisms including cell cycle blocking, induction of autophagy, promotion of Bax/Bcl-2 ratio and capase-3 level and depletion of ROS production. These data suggest that sodium selenite and G-Rh2 combination maybe a promising therapeutic approach for colorectal cancer treatment. In addition, by reducing drug concentration, drugs of combination may greatly decrease the systemic side effects in patients. However, more studies need to be accomplished. However, there are still a large number of in vitro and in vivo studies that need to be accomplished before entering the further clinical trials.

Authors Contribution

Changlai Zhu and Fang Liu contributed equally to this work.

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Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to subject of this article.

Ethical approval: *Not needed.*

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