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## Anti-proliferation effect of zoledronic acid on human colon cancer line SW480

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## ABSTRACT

**Objective:** To investigate the anti-proliferation effect and mechanism of zoledronic acid (ZOL) on human colon cancer line SW480.**Methods:** SW480 cells were treated with 0, 12.5, 25, 50, 100 and 200  $\mu\text{mol/L}$  of ZOL for 48 h, and CCK-8 assay was employed to obtain the survival rate of SW480 cells. SW480 cells were treated with 25  $\mu\text{mol/L}$  of ZOL for 0, 12, 24, 48 and 72 h, and then the survival rate was obtained. SW480 cells of the ZOL group were treated with 25  $\mu\text{mol/L}$  of ZOL for 48 h, while cells of the CsA + ZOL group were pretreated with 10  $\mu\text{mol/L}$  of CsA for 0.5 h and then treated with 25  $\mu\text{mol/L}$  of ZOL for 48 h. Then the survival rates of SW480 cells of the control group, ZOL group and CsA + ZOL group were determined. Flow cytometry was employed to detect the apoptosis rate and the mitochondrial transmembrane potential ( $\Delta\Psi\text{m}$ ) of the three groups and Western blot was used to detect the expressions of cyt C in the cytosol of the three groups.**Results:** ZOL inhibited the proliferation of SW480 cells, and the inhibition rate positively correlated with the concentration of ZOL and the action time ( $P < 0.01$ ). The cell survival rate and the  $\Delta\Psi\text{m}$  of the ZOL group were greatly lower than those of the control group, while the apoptosis rate and the expression of cyt C in the cytosol were obviously higher than those of the control group. All the differences showed distinctly statistical significances ( $P < 0.01$ ). The cell survival rate and the  $\Delta\Psi\text{m}$  of the CsA + ZOL group were all lower than those of the control group, but substantially higher than those of the ZOL group; while the apoptosis rate and the expression of cyt C in the cytosol were higher than those of the control group, but distinctly lower than those of the ZOL group. All the differences were statistically significant ( $P < 0.01$ ).**Conclusions:** ZOL can induce the apoptosis in human colon cancer line SW480 and then inhibit the proliferation of SW480 cells directly by opening the mitochondrial permeability transition pore abnormally, decreasing  $\Delta\Psi\text{m}$ , and releasing the cyt C into the cytosol. And the effect enhances with the increases of the concentration of ZOL and the action time.

## 1. Introduction

In recent years, the transformation to delicate diet structure and intensive life style cause rapidly increase of colorectal

cancer morbidity by nearly 5% every year, which far exceeds 2% of the international average standard [1]. The latest investigation report on cancer epidemiology showed that the morbidity and mortality rates of colorectal cancer in 2010 in our country reached 16.14% and 7.55%. Among them, most patients were males. The morbidity and mortality of male patients were 18.75% and 9.10%, respectively [2]. Colorectal cancer has obviously become one of the most dangerous killers for human health which severely threatens people's life and health. The grim situation has set off an alarm bell for medical workers to attach importance to the treatment research of the disease. As the newest nitrogen-containing

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bisphosphonate, the bone suppression absorption activity of zoledronic acid (ZOL) increases by 2000 times as compared to that of the flexor chloride sodium phosphate and reaches the 200 times of that of pamidronate [3], which is widely employed to prevent and treat solid tumor bone metastases and delay the development of the cancer-caused bone damage. In the further clinical experiences, it is proved that ZOL can not only prevent bone diseases, but also have effects on the cancer activity which can inhibit the proliferation and reduce the activity of cancer cells. However, researches on effects of ZOL were mainly on diseases, such as breast cancer, prostate cancer, squamous-cell carcinoma and so on [4–6]. There are few researches on the effects of ZOL on colorectal cancer. Therefore, the study aims to propose a new development direction for the treatment of colorectal cancer by investigating the anti-proliferation effect and mechanism of ZOL on human colon cancer line SW480, and now it is reported as follows.

## 2. Materials and methods

### 2.1. Cell lines, experimental materials and methods

Six concentration levels of ZOL were established and every level was treated with three holes parallel sample for subculture until the concentration of the logarithmic phase of SW480 (Shanghai Cells of Chinese Academy of Sciences) became  $2 \times 10^4$ /mL after digestion and dilution. They were placed on 96-well plates with 0.1 mL/well for anchorage growth under a suitable environment. After 24 h, the medium was changed into a fresh medium which had ZOL (Novartis Co. Ltd, Switzerland) and the concentrations of ZOL of each hole were 0, 12.5, 25, 50, 100 and 200  $\mu\text{mol/L}$ . After 48 h of the infiltration with ZOL, 0.01 mL of CCK-8 reagent (Shanghai LI Rui Biotechnology Co., Ltd.) was added along the hole wall into each hole. The reagent and the medium were mixed by taping the culture plate. After 4 h of full reaction, the light absorption values at 450 nm of each group were detected. The experience was conducted three times to investigate the effects of ZOL on anti-proliferation of SW480 in different concentrations.

Five time levels were established and meanwhile parallel sample with three holes in each level was treated. SW480 cells in the logarithmic phase with the concentration of  $2 \times 10^4$  mL were cultured on 96-well plates with 0.1 mL/well. After adherent culture, the medium was changed into a fresh medium and then they were continuously cultured for 72 h. Then in the corresponding time points, 25  $\mu\text{mol/L}$  of ZOL was added into them. After ZOL was infiltrated for 0, 12, 24, 48 and 72 h, CCK-8 reagent was added. After 4 h of full reaction, the light absorption values at 450 nm of each group were detected. The experience was conducted three times to investigate the effects of ZOL on anti-proliferation of SW480 in different time periods.

Three control groups were formed and meanwhile parallel sample with three holes was treated. SW480 cell suspension with the concentration of  $4 \times 10^5$ /mL was inoculated on 6-well plates for anchorage growth under a suitable environment. After 24 h, the medium of the CsA + ZOL group was changed into a fresh medium which contained 10  $\mu\text{mol/L}$  of cyclosporine A for pretreatment. The media of the control and the ZOL groups were changed into equal number of fresh media. After CsA had infiltrated 0.5 h, 25  $\mu\text{mol/L}$  of ZOL was respectively added into the ZOL and CsA + ZOL groups. The three groups were continuously cultured for 48 h, and then they were proceeded as

follows: ① 0.01 mL of CCK-8 reagent was added along the hole wall into each hole. After 4 h of full reaction, the cell activity was tested in the light of the CCK-8 kit instruction to study the differences of the survival rate of SW480 cells; ② cells treated with digestion, washing, dilution and other operations were collected and their apoptosis rates were determined by FACS-Calibu flow cytometry (BD Company, USA); ③ suspensions of each group which had made form cell's digestion and washing were collected and fully mixed with 1  $\text{mmol/L}$  of JC-1 (Shanghai Yisheng Biotechnology Co., Ltd.). Then after shading cultivation for 20 min, cells were collected by centrifugation. After washing and diluting many times, the fluorescence intensity was detected by flow cytometry to investigate the difference of the mitochondrial transmembrane potential ( $\Delta\Psi\text{m}$ ); ④ cells after homogenate of each group were centrifuged at 12000 g for 15 min at 4 °C. Then, the supernatant was collected and its expression quantity of cyt C was tested by Western Blot to investigate the differences of the content of cyt C in the cytosol in each group.

### 2.2. Statistical methods

SPSS 19.0 was used for statistical analysis. Cell survival rate, apoptosis rate,  $\Delta\Psi\text{m}$ , and the protein content of cyt C were all represented with mean  $\pm$  SD. As for the data of the cell survival rates which were treated with ZOL in different concentration levels and time levels, ANOVA was used to analyze multi-group comparison and *SNK-q* was used to test comparison between two groups. As for the data of the control groups, comparison between two groups was tested by *t*-test.  $P < 0.05$  showed statistical significance.

## 3. Results

### 3.1. Dose-dependent effects of zoledronic acid on SW480 cell survival rate

The cell survival rate which had detected in the concentration of 0  $\mu\text{mol/L}$  of ZOL was regarded as the reference standard. The cell survival rates of SW480 cells which were treated with 12.5, 25, 50, 100 and 200  $\mu\text{mol/L}$  of ZOL for 48 h dropped to  $(64.23 \pm 3.23)\%$ ,  $(51.88 \pm 3.06)\%$ ,  $(44.81 \pm 2.93)\%$ ,  $(39.28 \pm 2.67)\%$  and  $(30.24 \pm 3.34)\%$  respectively. With the increase in the infiltrating concentration of ZOL, the survival rates of SW480 in its different concentration levels decreased step by step, and the comparison differences had distinctly statistical significances ( $P < 0.01$ ). Compared with the untreated group, cell survival rates of SW480 cells in the different concentration levels of ZOL were significantly lower. Also, the comparison differences had distinctly statistical significances ( $P < 0.01$ ) (Table 1).

### 3.2. Time-dependent effects of zoledronic acid on SW480 cell survival rate

The cell survival rate of ZOL which had detected after infiltrating for 0 h was regarded as the reference standard. The cell survival rates of SW480 cells which were respectively treated with 200  $\mu\text{mol/L}$  of ZOL for 12, 24, 48 and 72 h dropped to  $(90.77 \pm 2.82)\%$ ,  $(73.38 \pm 2.49)\%$ ,  $(51.88 \pm 3.06)\%$  and  $(26.25 \pm 1.79)\%$ . With the extension of the infiltration time in

**Table 1**

Dose-dependent effects of zoledronic acid on SW480 cell survival rate.

Concentration of ZOL ( $\mu\text{mol/L}$ )	Cell survival rate (%)	Inhibition rate (%)
0	101.05 $\pm$ 3.03	0
12.5	64.23 $\pm$ 3.23 <sup>(1)</sup>	36.44
25	51.88 $\pm$ 3.06 <sup>(2)</sup>	48.66
50	44.81 $\pm$ 2.93 <sup>(3)</sup>	55.66
100	39.28 $\pm$ 2.67 <sup>(4)</sup>	61.13
200	30.24 $\pm$ 3.34 <sup>(5)</sup>	70.07
<i>P</i>	0.000	–
<i>F</i>	295.8	–

Compared with the ZOL group with a concentration of 0  $\mu\text{mol/L}$ : (1)  $P = 0.000$ ,  $q = 24.942$ , (2)  $P = 0.000$ ,  $q = 34.254$ , (3)  $P = 0.000$ ,  $q = 40.029$ , (4)  $P = 0.000$ ,  $q = 45.885$ , (5)  $P = 0.000$ ,  $q = 47.106$ .

ZOL, the survival rates of SW480 in its different time levels decreased step by step, and the comparison differences had distinctly statistical significances ( $P < 0.01$ ). The cell survival rates of SW480 cells in the different infiltration time levels in ZOL were significantly lower than those of the untreated groups. Also, the comparison differences had distinctly statistical significances ( $P < 0.01$ ) (Table 2).

### 3.3. Comparison of cell survival rate, apoptosis rate, $\Delta\Psi_m$ and the expression quantity of cyt C in the cytosol of the three groups

The detected cell survival rate, apoptosis rate,  $\Delta\Psi_m$  and the expression quantity of cyt C in the cytosol of the control group were considered as the reference standards. Compared with the numerical values of the control group, the cell survival rate of the ZOL group decreased significantly accompanying with the obvious increase of cell apoptosis rate and the expression quantity of cyt C in the cytosol and the decrease of  $\Delta\Psi_m$  meanwhile. The differences had distinctly statistical

**Table 2**

Time-dependent effects of zoledronic acid on SW480 cell survival rate.

Infiltrating time of ZOL (h)	Cell survival rate (%)	Inhibition rate (%)
0	100.83 $\pm$ 1.86	0
12	90.77 $\pm$ 2.82 <sup>(1)</sup>	9.98
24	73.38 $\pm$ 2.49 <sup>(2)</sup>	27.22
48	51.88 $\pm$ 3.06 <sup>(3)</sup>	48.55
72	26.25 $\pm$ 1.79 <sup>(4)</sup>	73.97
<i>P</i>	0.000	–
<i>F</i>	315.7	–

Compared with the group infiltrating in ZOL for 0 h: (1)  $P = 0.000$ ,  $q = 9.822$ , (2)  $P = 0.000$ ,  $q = 27.461$ , (3)  $P = 0.000$ ,  $q = 41.846$ , (4)  $P = 0.000$ ,  $q = 87.836$ .

**Table 3**Comparison of cell survival rate, apoptosis rate,  $\Delta\Psi_m$  and the expression quantity of cyt C in the cytosol of the three groups.

Groups	Cell survival rate (%)	Apoptosis rate (%)	$\Delta\Psi_m$ (%)	Cyt C (%)
Control group	100.05 $\pm$ 2.77	2.31 $\pm$ 1.19	98.95 $\pm$ 2.06	100.01 $\pm$ 1.84
ZOL group	53.24 $\pm$ 3.31 <sup>(1)</sup>	16.88 $\pm$ 1.92 <sup>(2)</sup>	40.52 $\pm$ 3.57 <sup>(3)</sup>	162.52 $\pm$ 4.22 <sup>(4)</sup>
CsA + ZOL group	81.92 $\pm$ 5.40 <sup>(5)(6)</sup>	9.33 $\pm$ 1.36 <sup>(6)(10)</sup>	80.22 $\pm$ 2.83 <sup>(7)(11)</sup>	137.51 $\pm$ 3.15 <sup>(8)(12)</sup>

Compared with the control group: (1)  $P = 0.000$ ,  $t = 32.536$ , (2)  $P = 0.000$ ,  $t = -19.350$ , (3)  $P = 0.000$ ,  $t = 42.528$ , (4)  $P = 0.000$ ,  $t = -40.735$ , (5)  $P = 0.000$ ,  $t = 8.962$ , (6)  $P = 0.000$ ,  $t = -11.654$ , (7)  $P = 0.000$ ,  $t = 25.300$ , (8)  $P = 0.000$ ,  $t = -30.839$ ; Compared with the ZOL group: (9)  $P = 0.000$ ,  $t = -13.584$ , (10)  $P = 0.000$ ,  $t = 9.627$ , (11)  $P = 0.000$ ,  $t = -26.143$ , (12)  $P = 0.000$ ,  $t = 14.248$ .

significances ( $P < 0.01$ ). Although the cell survival rate of the CsA + ZOL group was significantly lower than that of the control group, it went up again compared with the ZOL group; its apoptosis rate and the expression quantity of cyt C in the cytosol were higher than those of the control group but accordingly lower than that of the ZOL group;  $\Delta\Psi_m$  of the CsA + ZOL group was nearly 20% lower than that of the control group, but compared with the ZOL group, it increased distinctly. Also, the differences had distinctly statistical significances ( $P < 0.01$ ) (Table 3).

## 4. Discussion

The statistical data of the International Agency for Research on Cancer showed that 1.4 million new cases of colorectal cancer were found only in 2012 in the world which accounted for 9.7% of the total number of patients diagnosed with cancer in that year. Colorectal cancer has already become the third common cancer in the whole world [7,8]. Although colorectal cancer is prevailing mostly in Europe and the United States, its morbidity and mortality rates have been increasing swiftly in China, especially colon cancer. The morbidity of colon cancer is significantly higher than that of the colorectal cancer [9]. Scholars predicted that the morbidity of colon cancer in 2015 would be doubled as compared to that in 2002, which would increase to 13% [10]. Confronting with the menacing disease, it is greatly significant and just around the corner to explore effective treatment methods.

Among those several bisphosphonates drugs in clinic, ZOL is given high attentions because of its strong efficacy and persistence. Besides, its performance in the treatment of cancer is also the greatest. *In vitro* experimental results showed that infiltration of 20  $\mu\text{mol/L}$  of ZOL could decrease the cell survival rate of MDA-MB-231 of breast cancer by 75%, which is the three times effect of pamidronate of the same concentration and 100  $\mu\text{mol/L}$  of flexor chloride sodium phosphate could show such anti-cancer activity. In addition, ZOL under the concentration of 100  $\mu\text{mol/L}$  has no side effects on normal fibroblasts [11]. This study showed that ZOL also has anti-proliferation effect on human colon cancer line SW480. Besides, the inhibition rate increases with the increase of infiltrating concentration and the extension of action time. Previous researches indicated that ZOL could prevent the isoprene from synthesis by reducing the activity of farnesyl diphosphate synthase. As a result, the modification and maturation of small G protein lack of its necessary material, which causes the transmission failure of the cell membrane channel signal and then cancer cell would lost their activity and even die [12].

Apoptosis is a kind of rigorous and orderly 'suicide' which is adopted autonomously when the body confronts adverse conditions. Researches pointed that mitochondria play an important

role in the process. The collapse of its structure and function of the cells may hasten the death of the cells [13,14]. In this study, after treated with 25  $\mu\text{mol/L}$  of ZOL for 48 h, the cell survival rate of SW480 decreased to  $(53.24 \pm 3.31)\%$ ; the apoptosis rate increased;  $\Delta\Psi_m$  decreased sharply and the content of cyt C increased significantly. All those indicated that the abnormal opening of the mitochondrial permeability transition (PT) pore which induces the apoptosis is closely related to the inhibition effects of ZOL on SW480 cells. PT pore penetrates the double membrane of the mitochondria. Under the normal state, it could limit the penetration of macromolecular substances. And cyt C protein is kept out of the gap of the internal and external mitochondria membranes and maintains the stable physiological potential difference of the internal and external mitochondria membranes, which is very important to the mitochondrial capacity [15]. It is predicted that ZOL destroys the mitochondrial membrane function structure of SW480. As a result, the PT pore opens abnormally and cyt C protein gushes into the cytoplasm, which causes the direct increase of the expression quantity of cyt C in the cytosol. And these cyt C is the exact cause for cell apoptosis by activating Caspase-series protease of cytoplasm [16,17]. At the same time, since the proton condition of the both sides of the membrane is broken,  $\Delta\Psi_m$  decreases disorderly and the composition of ATP is forced to stop. Cells enter their energy depletion countdown. In the control group, although SW480 cells pretreated with Mitochondrial PT pore blocking agent CsA and infiltrated with ZOL still show a serious of apoptosis phenomena which are mediated by mitochondria and growth inhibition, it goes up again as compared to the  $\Delta\Psi_m$  group. The content of cyt C in the cytosol decreases and the apoptosis rate improves distinctly. All these further confirm the prediction that ZOL triggers the damage of the mitochondrial membrane and induces the apoptosis of SW480, while the specific truncation effect of CsA on the opening of PT pore can effectively put off the collapse of the mitochondria and the growth inhibition of cells.

In addition, it also appeared in the experience of breast cancer and osteosarcoma cell that ZOL could facilitate the apoptosis of the cancer cells, but it was revealed in the experience targeting of mouse pulmonary adenocarcinoma and human cholangiocarcinoma cell that though ZOL inhibits the proliferation of cells, it did not induce apoptosis [18,19], which indicated that ZOL had more than one kind of anti-cancer mechanism. Li *et al.* reported that ZOL could delay cell proliferation by preventing lung cancer cells from entering the G<sub>2</sub>-M period [20].

In conclusion, ZOL can facilitate the apoptosis in human colon cancer line SW480 and then inhibit the proliferation of SW480 cells directly by opening the mitochondrial permeability transition pore abnormally, decreasing  $\Delta\Psi_m$ , and releasing the cyt C into the cytosol. Further in-depth explorations may hopefully make ZOL a winning weapon in the battle of the treatment of colorectal cancer.

### Conflict of interest statement

We declare that we have no conflict of interest.

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