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Original Research Article

Effect of sinomenine on proliferation and apoptosis of oral squamous cell carcinoma cells, and its underlying mechanism of action

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Abstract

Purpose: *To investigate the effect of sinomenine on the proliferation and apoptosis of oral squamous cell carcinoma cells (OCSSs), and its underlying mechanism of action.*
Methods: *CCK-8 method was used to determine the inhibitory effects of different concentrations of sinomenine (50, 100, 200, 400 and 800 ug/mL) on proliferation of CAL-27 cells on treatment for 24 and 48 h. Cell migration was assayed using scratch test, while cell cycle was measured by flow cytometry. Hoechst 33258 fluorescence staining was conducted to determine apoptotic morphology. The effect of sinomenine on the expressions of cyclinD1, cyclinD3, cyclin-dependent kinases-2 (CDK2), Bax, B-cell lymphoma/leukmia-2 (Bcl-2), Caspase-3 and Caspase-9 was determined by western-blotting (WB).*
Results: *Sinomenine significantly inhibited proliferation and migration of CAL-27 cells in a concentration-dependent manner, but not in a time-dependent fashion. It inhibited the transition from G0/G1 phase to S phase in CAL-27 cells. Sinomenine treatment also caused dispersion of CAL-27 cells. Moreover, the nuclei were pyknotic, and the cells translucent, indicating typical features of apoptotic morphology. Expressions of Bax, caspase 3 and caspase 9 protein were significantly up-regulated, while expressions of Bcl-2, cyclinD1, cyclinD3 and CDK2 protein were down-regulated by sinomenine.*
Conclusion: *Sinomenine induces apoptosis of OSCCs, and inhibits their proliferation and migration via a mechanism associated with up-regulation of Bax, Caspase-3 and Caspase-9, and down-regulation of Bcl-2.*

Keywords: *Sinomenine, Oral squamous cell carcinoma, Proliferation, Apoptosis, Mechanism*

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INTRODUCTION

Oral cancer is a common malignant tumor of the head and neck, accounting for 2.1 % of systemic malignancies [1]. Oral squamous carcinoma (OSC) accounts for more than 90 % of oral cancers [2]. At present, the treatment of OSC is based mainly on chemotherapy. However, serious drug resistance and side effects have been reported. Thus, it is essential to discover

natural bioactive compounds with low toxicity for effective inhibition of OSC.

Sinomenine is the main bioactive component of *Sinomenium acutum* (family Menisperrnaceae) [3]. Studies have shown that sinomenine exerts many pharmacological effects, including anti-arrhythmia, analgesia and anti-inflammatory effects [4-6]. Sinomenine inhibits breast cancer, lung cancer, liver cancer and other tumors, and has great clinical potential as an anti-tumor agent [7]. However, not much is known about the effect of sinomenine on OSC.

This study was carried out to investigate the effect of sinomenine on the proliferation and apoptosis of OSC CAL-27 cells, and the mechanism involved, so as to provide some evidence for its clinical application.

EXPERIMENTAL

Main reagents and instruments

Human OSC cell line CAL-27 was purchased from Zhengzhou Yizeng Company (Zhengzhou, China). Sinomenine was purchased from Baishikai Technology Company (Shanghai, China). Fetal bovine serum (FBS) and DMEM medium were purchased from Gibco, while CCK-8 kit was bought from Qihai Futai Co. Ltd, China. Rabbit Bcl-2, Bax, caspase-3 and caspase-9 antibodies were purchased from Cell Signaling. The instruments used were chemiluminescence instrument (Tanon, China); western blotting system (Bio-RAD, USA); carbon dioxide incubator (Thermo, USA), and inverted fluorescence microscope (Olympus, Japan).

Cell culture

Human OSC cell line CAL-27 was cultured in a 37 °C, 5 % CO_2, saturated humidity incubator in DMEM containing 10 % FBS. The experiments were performed on logarithmic growth phase cells.

Evaluation of cell viability

Logarithmic growth phase CAL-27 cells were digested with 0.25 % trypsin, and then dispersed in a complete medium in 96-well plates at a density of about 5 x 10^3 cells/well. The wells were treated with different concentrations of sinomenine (50, 100, 200, 400 and 800 ug/mL), with 6 replicate wells for each concentration. Each plate contained blank and control wells. Before the end of the culture (2 h), 10 uL CCK-8 was added to each well. The absorbance of each well was measured at a wavelength of 450 nm on an enzyme-linked immunosorbent detector. Cell viability (C) for each experimental group was measured relative to the normal control group, using Eq 1.

C (%) = (At/An)100 …… (1)

where At and An are the absorbance of treatment and normal groups, respectively.

Scratch test

CAL-27 cells in logarithmic growth phase were inoculated into 6-well plates at a density of 5×10^5 cells/well. At adherence, an aseptic pipette tip was used to make scratches at the bottom of the plate. After washing with PBS, the transverse width was measured under inverted fluorescence microscope. Based on the results of CCK-8 assay, sinomenine concentrations of 50, 100, 200, 400 and 800 ug/mL were used to treat the cells. Cells in each group were observed in 3 selected fields of view. The distances between scratches were measured with a caliper on an inverted microscope. The cell migration distance was estimated as the difference between the initial scratch width and the final scratch width.

Flow cytometry

The CAL-27 cells in logarithmic growth phase were inoculated in 6-well plate. Sinomenine at the final concentrations of 50, 100, 200 and 400 ug/mL was added to the cells. After another 24 h of culture in an incubator, the cells were digested and centrifuged for 5 min at 1,000 rpm. The supernatant liquid was discarded, and the cells were washed twice with pre-cooled PBS solution. Then, 1mL of pre-cooled 70 % ethanol was added to preserve the cells overnight at 4 ℃. Thereafter, flow cytometry was used to determine cell cycle. The experiment was repeated three times.

Bis-benzimide H 33258 staining

The CAL-27 cells in logarithmic growth phase were cultured for 24 h in 96 well plates at a density of 5 × 10^4 cells/well. Thereafter, they were treated with sinomenine at final concentrations of 100, 200 and 400 ug/mL, with 6 replicate wells for each concentration. The cells were cultured for 24 h in an incubator, after which the upper culture medium was discarded, and the cells were washed thrice with PBS solution. Then, 100 uL of Hoechst 33258 staining solution was added to each well, followed by incubation at 37 ℃ for 30 min in the dark. Excess staining solution was discarded, and the cells were washed twice with PBS. Then, the

morphology of the apoptotic cells in each group was observed under a fluorescence microscope.

Western blotting

Total protein was extracted from each group using cell lysis buffer, and the protein was subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane, and the membrane was blocked with skim milk solution at room temperature for 2 h. Thereafter, the membrane was incubated at room temperature for 2 h with primary antibodies for cyclin D1, cyclin D3, CDK 2, Bacl-2, Bax, caspase-3 and aspase-9. Then, the membrane was washed thrice with TBST, and incubated with secondary antibody for 1 h at room temperature, followed by washing 4 times with TBST. Finally, the bands were quantitatively analyzed using Image J image analysis software.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Two-group comparison was done with *t*-test, whole one-way ANOVA was used for comparison among groups. Multiple comparisons were carried out using Least Significant Difference (LSD). All the data analyses were done using SPSS version 21.0. Statistical significance was assumed at $p > 0.05$.

RESULTS

Sinomenine inhibited proliferation of CAL-27 cells

Compared with the control group, the proliferation of CAL-27 cells was inhibited for 24 h and 48 h by different concentrations of sinomenine. Cell proliferation was significantly inhibited in a concentration- and time-dependent fashion by sinomenine at a dose of 100 ug/mL for 24 h ($p < 0.05$). However, there was no statistically significant difference in inhibition of proliferation between cells treated for 48 h and 24 h with the same concentration of sinomenine ($p > 0.05$). These results are shown in Figure 1.

Sinomenine inhibited migration of CAL-27 cells

Compared with the control group, the area of scratch was significantly increased after cells were treated with sinomenine for 12 h ($p < 0.05$). However, after cells were treated with sinomenine for 24 h, there was no significant difference in the areas of scratch, when compared with 12-h treatment ($p > 0.05$), although the cells healed well. On treatment of the cells with different concentrations of sinomenine for 24 h, the area of cell scratch was significantly different from that of the control group ($p < 0.01$), indicating that cell migration was significantly inhibited by sinomenine. These results are shown in Table 1.

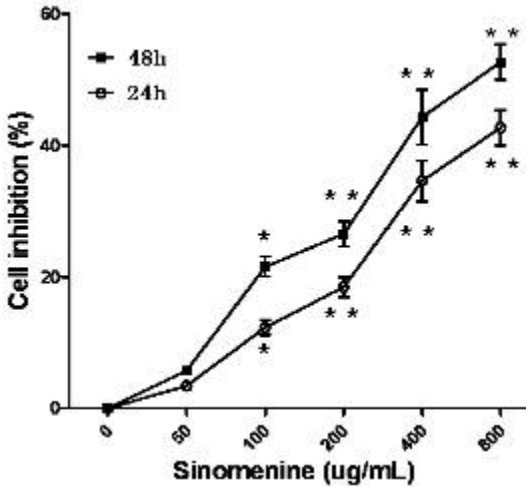

Figure 1: Inhibitory effect of sinomenine on CAL-27 cells. Data are presented as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$

Effect of sinomenine on cell cycle of CAL-27 cells

Table 2 shows that compared with the control group, sinomenine treatment increased the percentage of G0/G1 and S phase cells in CAL-27 cells after 24 h ($p < 0.05$), but had no significant effect on the percentage of cells in G2/M phase.

Table 1: Cell scratch area ratio (mean ± SD，n = 3)

Group	0 h	6 h	12 h	24 h
Control	1 ± 0	0.82 ± 0.01	0.36± 0.02	0.07 ± 0.03
50	1 ± 0	0.80 ± 0.03	0.37 ± 0.04	0.10 ± 0.05
100	1 ± 0	0.78 ± 0.09	0.42 ± 0.01	0.25 ± 0.06**
200	1 ± 0	0.75 ± 0.11	0.45 ± 0.05*	0.30 ± 0.08**
400	1 ± 0	0.70 ± 0.06	0.49 ± 0.10*	0.34 ± 0.12**
800	1 ± 0	0.68 ± 0.12	0.56 ± 0.12**	0.44 ± 0.10**

*p <0.05，**p <0.01

The results indicate that sinomenine significantly and concentration-dependently blocked the transition of G0/G1 phase cells to S phase, thereby inhibiting cellular growth.

Effect of sinomenine on expressions of cyclin-D1, cyclin-D3 and CDK2 proteins

As shown in Figure 2, the protein expressions of cyclin D1, cyclin D3 and CDK2 decreased significantly in CAL-27 cells treated with sinomenine at different concentrations, relative to control group. The GDPDH gene was used as endogenous control.

Effect of sinomenine on apoptosis of CAL-27 cells

Figure 3 shows that there were no significant differences in morphology, distribution and fluorescence color of CAL-27 cells treated with 100ug/ml sinomenine, when compared with the control group. However, treatment with 200 and 400 ug/mL sinomenine led to significant reductions in the number of cells. Moreover, the cells were translucent and scattered, with partial nuclear fragmentation and pyknotic nuclei, all of which are typical morphological features of apoptosis.

Effect of sinomenine on the protein expressions of Bacl-2 and Bax

Compared with the control group, the expression of Bcl-2 was significantly decreased after incubation of CAL-27 cells with different concentrations of sinomenine, while the expressions of Bax protein was significantly increased. These results are shown in Figure 4.

Sinomenine activated caspase-associated proteins

Caspase-3 and Caspase-9 related proteins were activated when CAL-327 cells were treated with sinomenine, relative to control (Figure 5). This suggests that the sinomenine-induced apoptosis of CAL-27 cells may be related to the activation of proteins associated with caspase pathway of apoptosis.

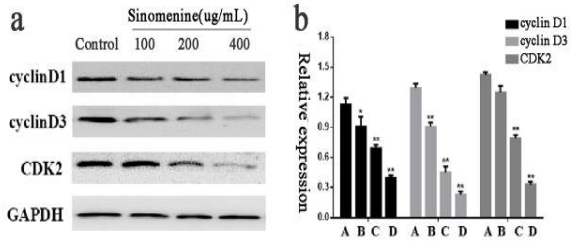

Figure 2: Protein expressions of cyclin D1, cyclinD3 and CDK2 in each group of CAL-27 cells. A: Control group, B: 100 ug/mL sinomenine group, C: 200 ug/mL sinomenine group, D: 400 ug/mL sinomenine group; *$p < 0.05$，**$p < 0.01$, compared to control group

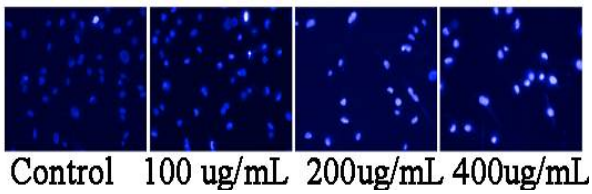

Figure 3: Features of apoptosis morphology in cells based on fluorescence staining

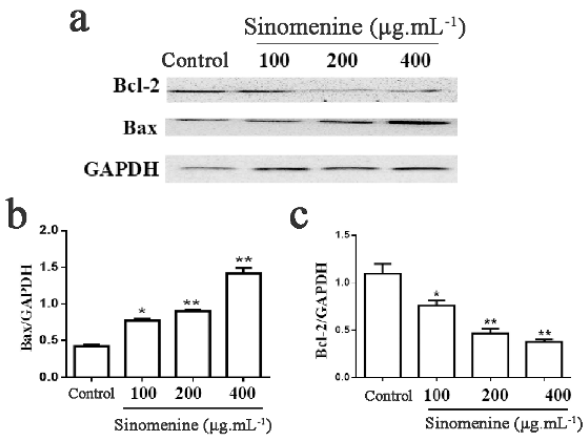

Figure 4: Protein expressions of Bax and Bcl-2 in each group of CAL-27 cells (mean ± SD，n = 3); *$p < 0.05$, **$p < 0.01$, compared with the blank group

Table 2: Effect of different concentrations of sinomenine on cell cycle of CAL-27 after 24 h intervention

Group	Concentration（ ug/ml)	G0/G1 phase (%)	S phase (%)	G2/M phase (%)
Control		65.2 ± 5.3	30.2 ± 3.0	2.9 ±2.3
Sinomenine	50	68.5 ± 1.8	28.6 ± 2.3	2.9 ± 3.1
	100	72.6 ± 2.5*	21.8 ±4.1**	5.6 ± 1.4
	200	75.1 ±3.5**	18.8 ± 5.2**	6.1 ± 1.3
	400	79.3 ± 4.2**	15.0 ± 2.8**	5.7 ± 0.9
	800	82.4 ± 6.7**	12.4 ± 1.9**	5.2 ± 1.6

*$P < 0.05$，**$p < 0.01$

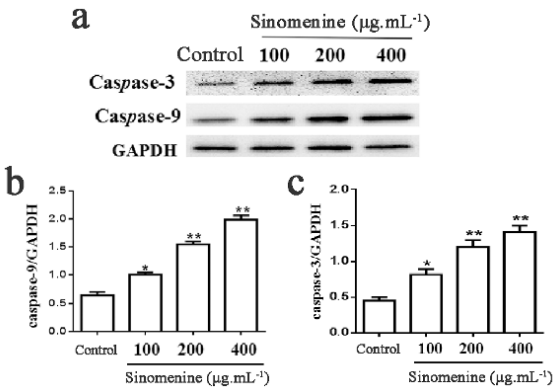

Figure 5: Effect of sinomenine on the protein expressions of caspase-3 and caspase-9 in CAL-27 cells. Data are presented as mean ± SD (n = 3). GDPDH was used as the endogenous control; **p* < 0.05，***p* < 0.01, relative to control group

DISCUSSION

Oral squamous carcinoma is the most common oral malignant tumor, accounting for more than 90 % of oral cancers. It is characterized by high probability of recurrence and rapid metastasis [8,9]. Sinomenine is an alkaloid extracted from the root and stem of *Sinomenium acutu*. Previous studies found that in addition to immunosuppression, anti-inflammation, anti-rheumatism and other effects, sinomenine is effective in the treatment of a variety of malignant tumors, and has a good prospect for clinical application [10]. In this study, the effect of different concentrations of sinomenine on the proliferation and migration of human OCSS cell line CAL-27 was investigated. The results showed that sinomenine inhibited the proliferation and migration of CAL-27 cells in a time-and dose-dependent manner.

The cell cycle is divided into G1 phase, S phase, G2 phase and M phase [11]. Its regulation is achieved by a series of important signaling molecules and cyclins. The primary cyclins include CDKs and CKIs [12]. Cyclin D, the main cell cycle protein in G1 phase, is activated by cell growth factor. At a certain threshold, cyclin D binds to, and promotes the expression of CDK2, activates the downstream substrates, and ultimately completes the cell cycle process [13]. The results obtained in the present study showed that treatment of CAL-27 cells with sinomenine for 24 h significantly increased the percentage of G0/G1 and S phase cells, but it appeared to have no effect on the percentage of G2/M phase cells. At the same time, the protein expressions of cyclin D subunit cyclin D1, cyclin D3 and CDK2 were down-regulated. These results demonstrate that the inhibitory effect of sinomenine on the proliferation of CAL-27 cells may be mediated through blockage of the cell cycle in G2/M phase, and down-regulation of protein expressions of cyclinD1, cyclinD3 and CDK2.

The Bcl-2 protein family is highly correlated with cell apoptosis. It is generally accepted that Bcl-2 prevents apoptosis, while Bax is responsible for inducing apoptosis [14,15]. Thus, Bcl-2 and Bax are a pair of important signal molecules that regulate apoptosis through the mitochondrial pathway. They regulate apoptosis by their effect on the activities of the caspase protein family [16]. Caspase-3 and caspase-9 are the most critical members of the caspase family [17]. Caspase-9 is an upstream initiator of apoptosis [18], while caspase3 is one of the most important apoptosis implementers in the caspase family. It plays a central role as a connecting link in the transmission of apoptotic signals [19]. In this study, it was found that sinomenine significantly induced apoptosis, nuclear fragmentation, pyknosis and aggregation in CAL-27 cells in a concentration-dependent manner. Moreover, sinomenine significantly up-regulated caspase3, caspase9 and Bax proteins, and down-regulated the protein expression of Bcl-2.

CONCLUSION

This study has demonstrated that sinomenine inhibits the proliferation of OSC CAL-27 cells and induces cell apoptosis via a mechanism involving up-regulation of the expressions of caspase3, caspase9 and Bax, and down-regulation of Bcl-2 expression. These findings provide an experimental basis for the clinical application of sinomenine in the treatment of oral squamous carcinoma.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors listed in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Tao Geng, Daxu Li ang Wei Cheng conceived and designed the study. Le Ren, Ran Xu, Zhe Zhang, Rui Wang collected and analyzed the data, while Tao Geng wrote the manuscript.

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