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Succinyl rotundic acid inhibits growth and promotes apoptosis in the HeLa cervical cancer cell line

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ABSTRACT

Rotundic acid (RA) is a kind of pentacyclic triterpene saponins, which widely exists in holly and other plants. It has anti-tumor and lipid-lowering effects, and has been widely used in the prevention and treatment of vascular diseases. Succinyl RA (SRA) is a kind of circular acid derivative with strong water solubility. However, SRA's effect and mechanism about the therapy of cervical cancer is still not clarified. The inhibitory effect of SRA on Hela cells and its molecular mechanism were investigated in this study. Hela cell line was used to establish an *in vitro* model to study the effect of SRA. The effects of SRA on cell proliferation, cell cycle and apoptosis were analyzed by flow cytometry and detected by 3-(4,5-dimethyl thiazole-2-yl)-2, 5-diphenyltetrazole bromide. The experimental results show that SRA can inhibit the growth of Hela and diffusion, inducing cell apoptosis. SRA can also reduce the expression of CyclinD 1 and p21. It was also decreased the expression of apoptosis inhibitor B cell lymphoma (*Bcl-2*), and increased the expression of *Bcl-2-related X-protein* and *Caspase-3*. This study provides evidence for the progress of SRA in inhibiting Hela cells and presents potential applications of SRA as an alternative therapy for cervical cancer.

Keywords: Succinyl Rotundic Acid, Cervical Cancer, Cell Proliferation, Apoptosis, Cell Cycle.

1. INTRODUCTION

Cancer is a serious life-threatening disease, the second leading cause of human death, just after cardiovascular disease [1–3]. Therefore, it is urgent to develop high efficient and low toxicitic anticancer drugs. Cervical cancer is the most common female cancer [4]. At present, the treatment of cervical cancer is hysterectomy and postoperative radiotherapy and chemotherapy. However, this treatment is not always effective [5]. Traditional Chinese medicine can regulate the function of normal organs, enhance the function of immune system and control tumor cells. The new direction of diagnosis and treatment of cervical cancer is the combination of traditional medicine and modern scientific methods.

Rotonic acid (RA), a pentacyclic triterpenoid acid [6–8], which possesses pharmacological activities such as antitumor and lipid-lowering effects, has been used to prevent and treat cardiovascular and cerebrovascular diseases [9–10]. However, the low polarity of RA [0.5% dimethyl sulfoxide (DMSO)], high fat solubility and low bioavailability limit the clinical application of RA. Many RA derivatives [11–13] have been synthesized by chemical methods, presenting higher polarity (0.1% DMSO) of succinyl (SRA) than RA and higher oral bioavailability in rats [14–16]. However, the effect of SRA on cervical cancer cells has not yet been evaluated. This study based on the research of the proliferation and apoptosis *in vitro*

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Hela, studies the influence of SRA on cervical cancer cell behavior and its molecular mechanism.

2. MATERIALS AND METHODS

2.1. Drugs

RA was isolated and purified from the dried bark of *Ilex rotunda*. SRA was obtained by chemical modification of RA. RA was heated in the presence of pyridine and reacted with succinic anhydride. Cooling to room temperature, after the completion of an action in ice water, dilute hydrochloric acid was applied to adjust the pH to 4∼5. After filtration, the filter residue was washed with water until it was colorless. The final SRA product was obtained by recrystallizing. The purity of SRA was 95.70% by high performance liquid chromatography (HPLC).

2.2. Cell Line

The cell lines, HeLa, C33A and SiHa were obtained from BeNa Culture Collection. RPMI 1640 Media (Gibco; Thermo Fisher Science, Inc.) FBS containing 10% FBS were cultured in a humid atmosphere with 5% CO_2 at 37 °C. HUVECs comes from Changchun Obstetrics and Gynecology Hospital, Media 199 (Gibco; Thermo Fisher Science, Inc.) Culture of 10% fetal bovine serum (GE health life sciences; HyClone). Exponential cells were used in subsequent experiments with humidifying in containing 5% CO_2 atmosphere, temperature of 37 °C.

2.3. Experimental Groups

Tetermination of the suitable concentration of SRA has been carried in the preliminary experiment. The patients were divided into 4 groups: DMSO (0.1%), low dose SRA (10 μmol/L, 0.1% DMSO), Medium dose group (20 μmol/L, 0.1% DMSO) and high dose group (40 μmol/L, 0.1% DMSO).

2.4. Cell Screening

HeLa, c33a, siha cells and huvecs were inoculated in 96-well plates with a density of 2×10^4 per well. After 24 hours of culturation, the cell culturation medium was discarded. The above treatment (200 μL) is in triplicate. After 48 hours of culturation, 20 μL ammonium bromide (μL) was used instead of culturation medium (5 mg/mL), Sigma-Aldrich (Sigma-Aldrich; Merck KGaA) was incubated with plate for 4 hours, the culturation medium was discarded, 150 μL of DMSO solution was added, stirred gently for 10 minutes, the absorbance intensity was decreased. The microplate reader was used to determinate the absorbance intensity at 490 nm (ELX-800; BioTek Instrument Co.). The cell viability was calculated with these absorbance values.

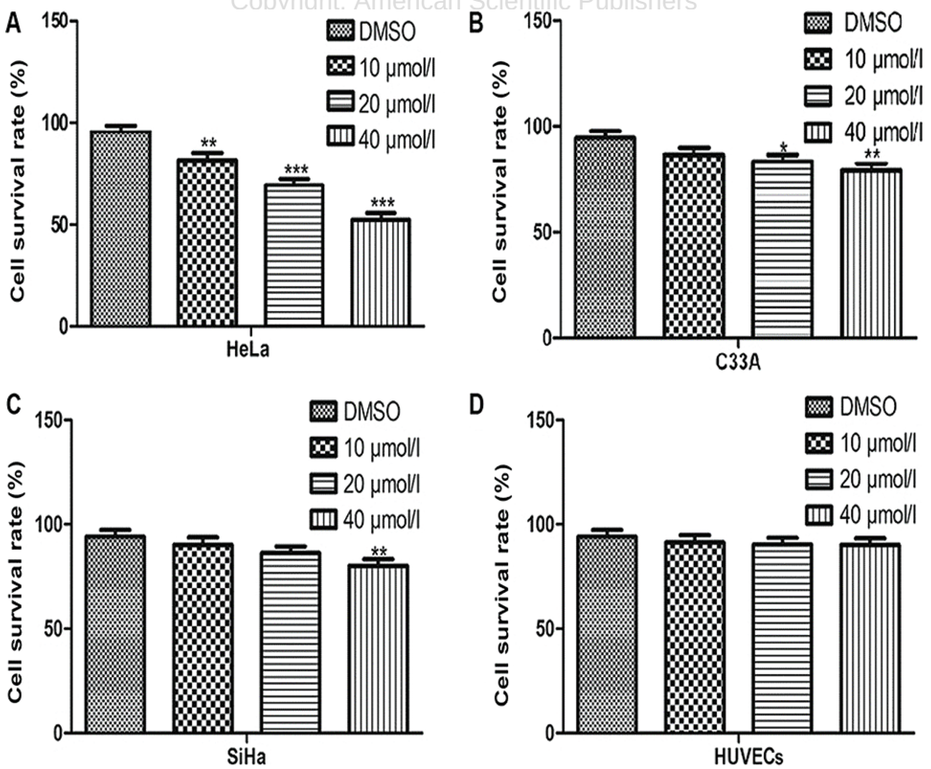

Fig. 1. Cell line selection, following treatment with succinyl rotundic acid, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in (A) HeLa, (B) C33A and (C) SiHa cells, and (D) HUVECs. Data are presented as the mean ± standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the DMSO group. DMSO, dimethylsulfoxide.

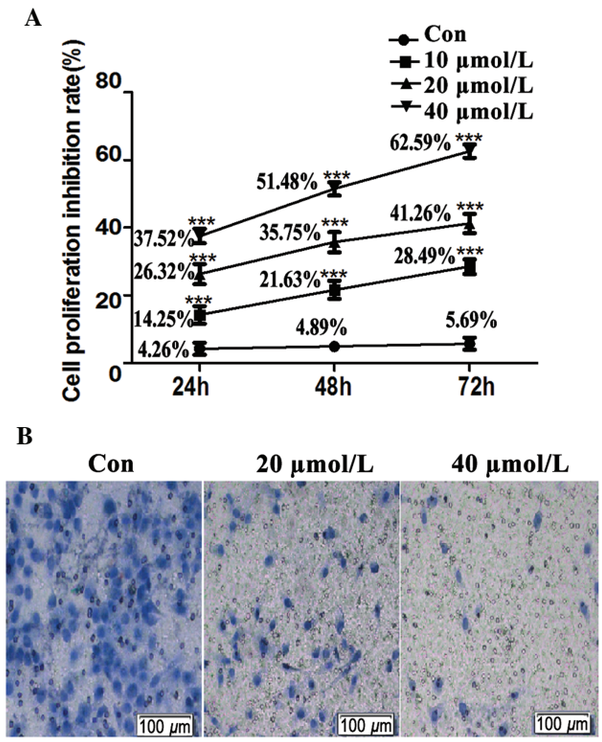

Fig. 2. Following treatment with succinyl rotundic acid, (A) HeLa cell proliferation was determined by MTT assay. (B) The transwell assay was used to determine cell invasiveness. Data are presented as the mean ± standard deviation; $^{***}P < 0.001$ compared with the DMSO group. Con, control; DMSO, dimethyl sulfoxide.

2.5. The Assay of Cell Proliferation

The density of the HeLa cells is 2×10^4 cells/well and it has been seeded in 96-well plates. According to the cell screening method, the cells were cultured for 24 h, 48 h and 72 h, and the cell proliferation was calculated by these absorbance values.

2.6. Invasion Assay

Logarithmic HeLa cells were harvested with 0.25% trypsin [without ethylenediamine tetraacetic acid (EDTA)] and diluted to 2×10^5 cells/mL in RPMI1640 medium containing 20% FET, bovine serum and above concentration of SRA. Each prepared HeLa cell (150 μL) was inoculated in the coated chamber of Matrigel (BD Bioscience) (Corning Life Science). The inferior cavity contained 600 μL of RPMI 1640 medium containing 20% fetal bovine serum and suitable SRA concentration. After 48 hours, the pure cells were treated with the permeable membrane, followed by washing with phosphate buffer solution (PBS) twice, being fixed for 20 minutes with paraformaldehyde, dyeing with 0.1% Cr, and crystallization for 30 minutes. Then each film was gently wiped with cotton swabs and rinsed with PBS 3 times. Membranes were randomly divided into 5 horizons, followed by microscope count each vision on the number of cells. Cell invasiveness was calculated by the number of cells passing through the membrane.

2.7. Westernblot Analysis

The total protein extraction kit (Beyotime) was used to extract the protein by lysing, collecting, and cracking

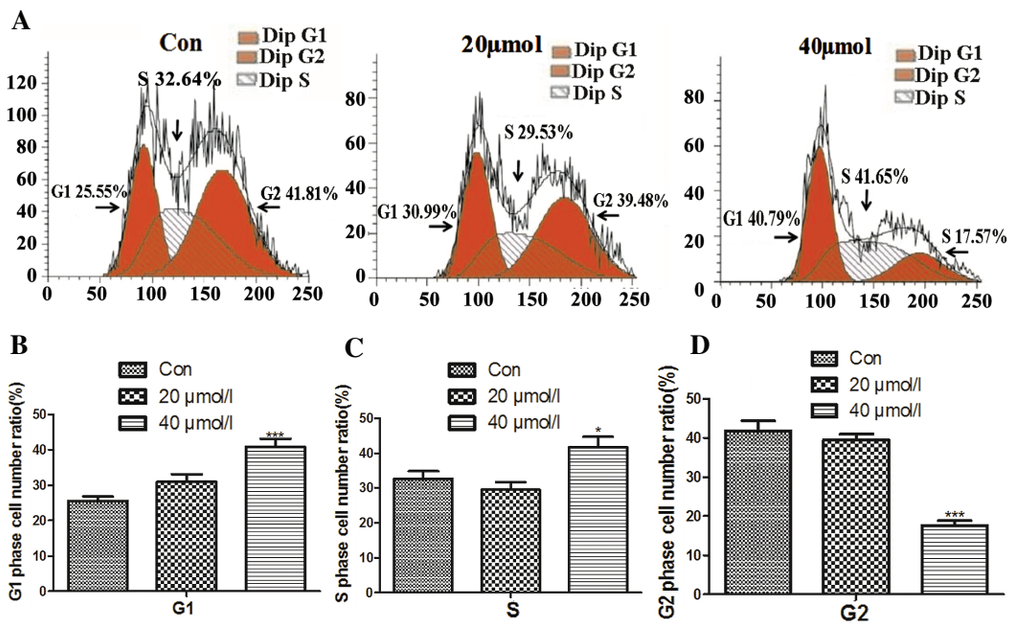

Fig. 3. Inhibition of the HeLa cell cycle following treatment with succinyl rotundic acid. (A) Flow cytometric analysis of the cell cycle. (B–D) Statistical analyses of the numbers of cells in (B) G1, (C) S and (D) G2 phases. Data are presented as the mean ± standard deviation; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the DMSO group. Con, control; DMSO, dimethylsulfoxide.

Article

the cells. The sample was then polymerized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (separation of 10% gel and 4% concentrate D glue). The protein samples were SDS-PAGE isolate and transferred to polyvinylidene fluoride membrane (EMD Millipore) and sealed with 5% bovine serum albumin at room temperature. The membrane was incubated with cyclin-D1, P21, B-cell-lymphadenoma-2(Bcl-2), Bcl-2-associated X protein (BAK), cleaved with caspase-3 and β-actin (1:500; BIOSS) overnight at 4 °C with primary antibodies, and subsequently washed with PBS containing Tween-20 for 3 times, 7 minutes each time. The membrane was then incubative. Secondary antibody was marked by horseradish peroxidase (1:5, 000; cat. no. A0208; Beyotime Biotechnology Institution) for 1 hour at room temperature. After this, PBST was used to clean membrane for 3 times, 7 minutes each time. Enhanced chemiluminescent substrate solutions (Solarbio Life Science) were then added to produce detectable signals. For image analysis, β-actin was applied as an interior control by the gel-pro-analyzer software (Media Cybernetics Inc.).

2.8. Flow Cytometric Analysis of Apoptosis

Cell suspension was added into the 6-well cell culturatioon plate, incubated for 24 hours, the supernatant was discarded, cell particles were collected, and washed twice with cold PBS 400 μL of 1× Annexin V and 5 μL of Annexin V-FITC, and incubated at the temperature between 2 °C−8 °C for 15 minutes in the darkness. Then, after staining with a liquid mixture, 10 μL of PI was

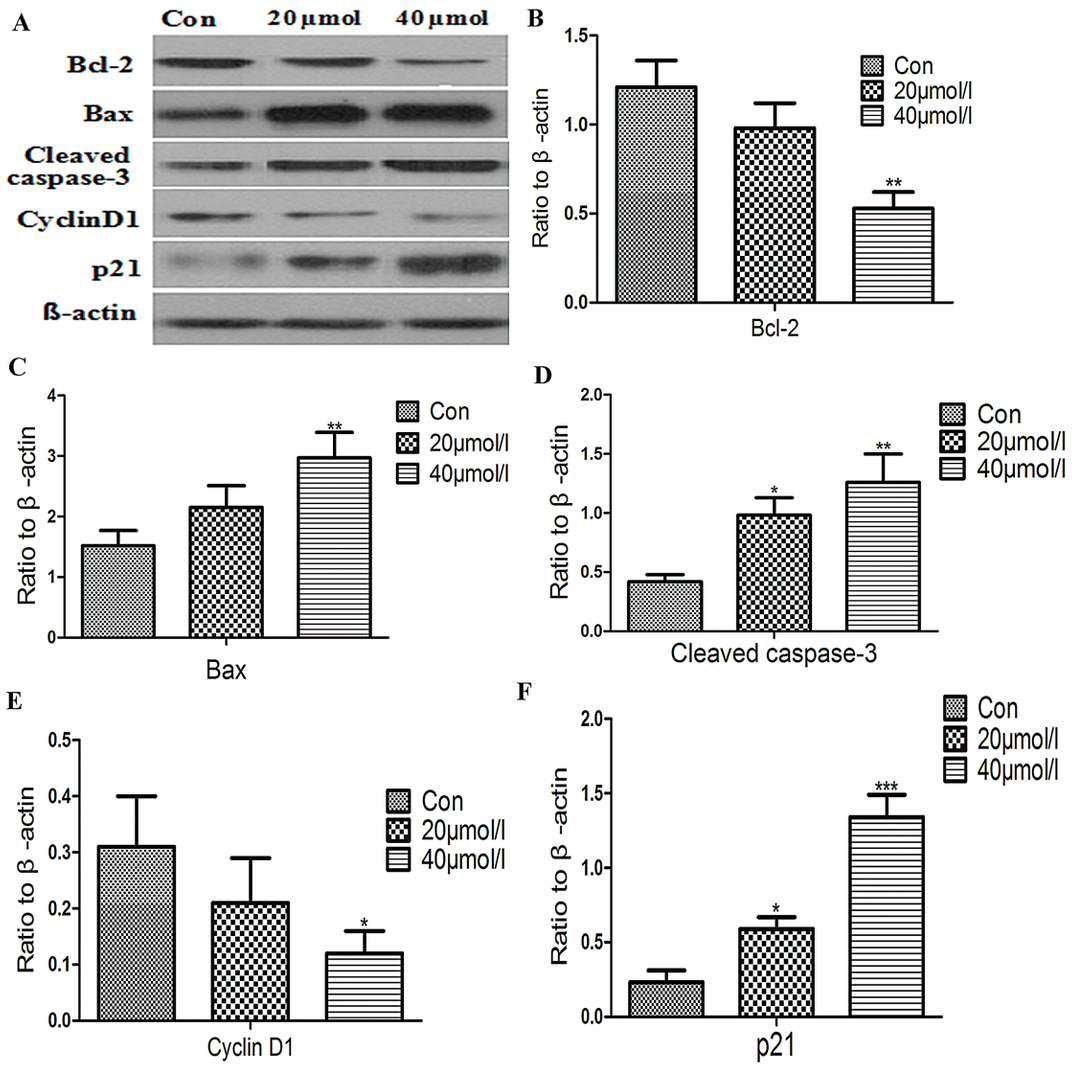

Fig. 4. Succinyl rotundic acid inhibits the expression of Bcl-2 and cyclin D1, and stimulates the expression of Bax, caspase-3 and p21 in HeLa cells. (A) Western blot analysis and quantitative assessment of the expression of (B) Bcl-2, (C) Bax, (D) caspase-3, (E) cyclin D1 and (F) p21. β-actin was used as the internal control for grayscale analysis. Data are presented as the mean ± standard deviation; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the DMSO group. Con, control; DMSO, dimethylsulfoxide; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

added. Cell apoptosis was detected by flow cytometry after ED treatment for 15 min at 2 °C–8 °C.

2.9. Flow Cytometry Analysis of Cell Cycle

After incubation with 90% of the density of collected cells, the cells were treated with 70% of cold ethanol, saved for 3 h at 4 °C, washed with PBS, and kept in the solution containing 25 μL of iodide organism and 10 μL of RNaseA dyeing buffer in suspended situation. The cell suspension of 37 °C in the dark was incubated 45 minutes using FACSC Alibur flow cytometry instrument (BD, Franklin Lake, NJ, USA) for the analysis of the cell cycle distribution. According to the different stages of DNA replication, the cell cycle was divided into four stages, G1, S, G2 and M. After PI staining, flow cytometry can detect changes in DNA content, so it can report the effects of treatment on the cell cycle.

2.10. Statistical Analysis

The data were expressed as mean ± standard deviation. Bonferroni post-hoc test was used for multiple comparison and One-way anova was used for inter-group comparison. Graphpad Prism 5.0 Software (Graphpad Software, Inc., La Jolla, CA, USA) was used to process data and numbers. When results $P < 0.05$, the difference was significant ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Cell Screening

MTT test showed different concentrations of SRA, 10×10^{-6} mol/L ($P < 0.01$), 20×10^{-6} mol/L ($P < 0.001$) and 40×10^{-6} mol/L ($P < 0.001$). SRA only had an inhibitory effect on C33A cells at 20 μmol/L ($P < 0.05$) and 40 μmol/L ($P < 0.01$) concentrations of SRA (Fig. 1(B)), while it had an inhibitory effect on SiHa cells at 40 μmol/L ($P < 0.01$; Fig. 1(C)). There was no inhibitory effect on HUVECs from SRA at any of the concentrations (Fig. 1(D)). The inhibitory effects for all cell lines were compared with the control (DMSO) for each concentration.

3.2. Inhibition of HeLa Cell Proliferation and Cell Cycle

Effects of diosgenin on proliferation, migration and invasion of glioma cells were studied by MTT, Transwell and cell cycle methods. The time points of inhibiting proliferation (10, 20 and 40 μmol/L SRA) were significantly different from DMSO group ($P < 0.001$; Fig. 2(A)). SRA-induced invasiveness data (19.0 ± 2.74) and (52.0 ± 4.23) of the control group (142.0 ± 4.15) were markedly different (Fig. 2(B), both $P < 0.001$). Cell cycle (FACS) analysis was performed (Fig. 3(A)). The results showed that the cell number of G1 phase and S phase increased in SRA (40 μmol/L) group ($P < 0.001$ and $P < 0.05$, respectively). The number of G2 cells was lower than the control group (Figs. 3(B–D)) ($P < 0.001$).

The levels of Bcl-2, Bax and cleaved caspase-3 in the different treatment groups were determined, and the results showed that the expression of Bcl-2 of 40 μmol/L SRA decreased by 44% compared with the control group ($P < 0.01$; Figs. 4(A and B)), the expression of Bax and cleaved-caspase-3 in the SRA group was 1.95 times ($P < 0.01$. Figs. 4(A and C)) and 3 times ($P < 0.01$; Figs. 4(A and D)) higher than that in the control group. The expression of cyclin D1 and p21 in SRA group was

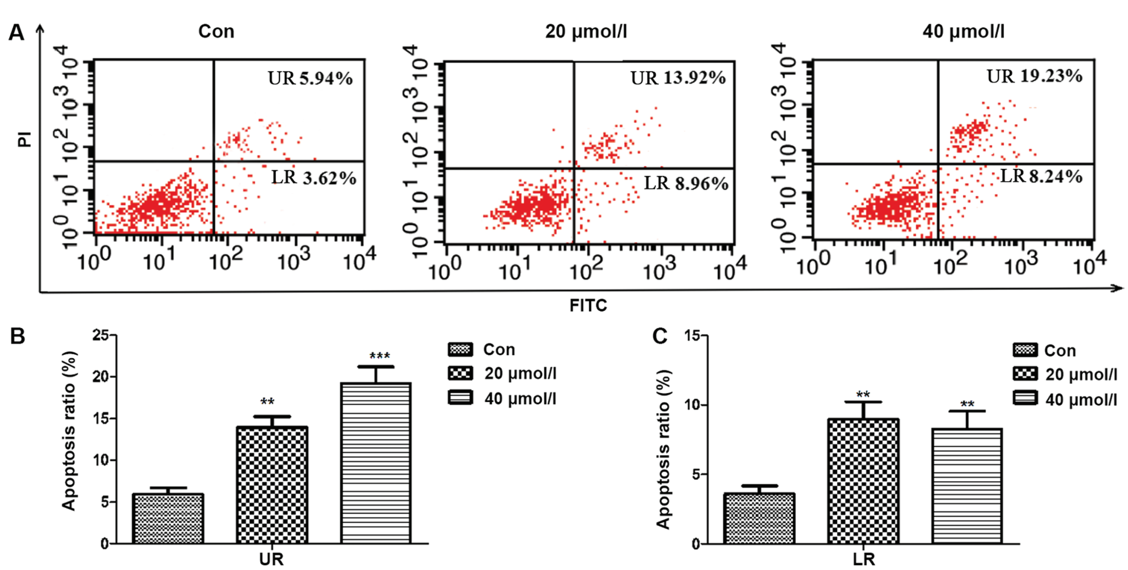

Fig. 5. Induction of HeLa cell apoptosis following treatment with succinyl rotundic acid. (A) Flow cytometric analysis of apoptosis. (B and C) The statistical analyses of cell apoptotic ratios in early (LR) and late apoptosis (UR). Data are presented as the mean ± standard deviation; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the DMSO group. FITC, fluorescein isothiocyanate, PI, propidium iodide; Con, control; DMSO, dimethylsulfoxide.

Article

0.39 times ($P < 0.01$. Figs. 4(A and C)) and 5.8 times ($P < 0.01$; Figs. 4(A and D)), respectively.

3.3. Induction of HeLa Cell Apoptosis by SRA

The results of flow cytometry in SRA group in comparison with the control group showed that the cellular apoptosis (Fig. 5(A)) in the SRA group (40 μmol/L) was 2.39- and 2.87-fold higher, respectively ($P < 0.001$ and $P < 0.01$; Figs. 5(B–C)).

Prior to a previous study by our group [17–19], there were no reports on the structure, synthesis methods and activity of SRA [20–23]. SRA is a novel compound [24]. The antitumor activity of RA derivatives has been demonstrated. In 5 cell lines (A-375, SPC-A1, HeLa, HepG2 and NCI-H446) [25–27], the effects of SRA on HeLa cells has not been studied, nor has its potential therapeutic approach. As a result, the benefits of cervical cancer are unclear [28–30]. Flow cytometry can determine the changes of DNA content in different stages of the cell cycle [31–32]. SRA prolonged G1 and S phases, reduced G2 phase, and reduced cell proliferation.

Apoptosis is cell death caused by both internal and external factors. Caspase 3 [33–35] is a kind of effective Caspase apoptosis in the last stage of the degradation of the cell. Pro-apoptotic protein Bax and cell survival promoter Bcl-2 [36] play key roles in the regulation of endogenous apoptosis signals. Meanwhile, p21 can regulate cell cycle and induce apoptosis.

4. CONCLUSION

In conclusion, this study on cervical cancer cell lines provided preliminary characteristics of SRA's influence, and these results demonstrated the potential application value of SRA in the treatment of cervical cancer.

Authors' Contributions

Minghua Duan and Hongbo Ye designed the study. Yufang He, Jiaming Sun, Minlun Nan, Xue Wang, He Lin were responsible for experiments. Guangfu Lv and Yong Li conducted experiments and analyzed data.

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References and Notes

1. Parkin, D.M., **2001**. Global cancer statistics in the year 2000. *The Lancet Oncology*, *2*(9), pp.533–543.
2. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A. and Jemal, A., **2018**. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), pp.394–424.
3. Gu, X., Zheng, R., Xia, C., Zeng, H., Zhang, S., Zou, X., Yang, Z., Li, H. and Chen, W., **2018**. Interactions between life expectancy and the incidence and mortality rates of cancer in China: A population-based cluster analysis. *Cancer Communications*, *38*(1), Article ID: 44.
4. Poureslami, I., Nimmon, L., Rootman, I. and Fitzgerald, M.J., **2017**. Health literacy and chronic disease management: Drawing from expert knowledge to set an agenda. *Health Promotion International, 32*(4), pp.743–754.
5. Lei, D.L., **2018**. Clinical effect of early surgical treatment of cervical cancer clinical effect of early surgical treatment of cervical cancer. *Journal of Practical Gynecologic Endocrinology, 34*(5), pp.78–79.
6. Li, J.J., Chen, F.F., Song, L.Y., Zhu, J.H. and Yu, R.M., **2017**. Study progress on antitumor activities of artemisinin and its derivatives. *Chinese Journal of Biochemical Pharmaceutics*, *37*(3), pp.10–14.
7. He, B., Chen, X.X., Li, J.H., Jiang, T. and Luo, J.P., **1997**. Effects of extract of cortex ilicis rotundae on cardiovascular pharmacology. *Journal of Chinese Medicinal Materials*, *20*(6), pp.303–306.
8. Hsu, Y.M., Hung, Y.C., Hu, L.H., Lee, Y.J. and Yin, M.C., **2015**. Anti-diabetic effects of madecassic acid and rotundic acid. *Nutrients*, *7*(12), pp.10065–10075.
9. He, Y.F., Nan, M.L., Sun, J.M., Meng, Z.J., Yue, F.G., Zhao, Q.C., Yang, X.H. and Wang, H., **2012**. Synthesis, characterization and cytotoxicity of new *rotundic acid* erivatives. *Molecules*, *17*(2), pp.1278–1291.
10. He, Y.F., Nan, M.L., Sun, J.M., Meng, Z.J., Li, W. and Zhang, M., **2013**. Design, synthesis and cytotoxicity of cell death mechanism of *rotundic acid* derivatives. *Bioorganic & Medicinal Chemistry Letters*, *23*(9), pp.2543–2547.
11. He, Y.F., Nan, M.L., Zhao, Y.W., Sun, W.Y., Li, W. and Zhao, Q.C., **2016**. Design, synthesis and evaluation of antitumor activity of new *rotundic acid* acylhydrazone derivatives. *Zeitschrift Für Naturforschung C*, *71*(5–6), pp.95–103.
12. Chen, Y., He, Y.F., Nan, M.L., Sun, W.Y., Hu, J., Cui, A., Li, F. and Wang, F., **2013**. Novel rotundic acid derivatives: Synthesis, structural characterization and in vitro antitumor activity. *International Journal of Molecular Medicine*, *31*(2), pp.353–360.
13. Nan, M.L., He, Y.F., Si, X.L., Zhao, Y.W., Wang, X., Bai, X. and Li, C.J., **2019**. Study on synthesis and identification of rotundic acid esters derivatives and in vitro antitumor activity. *China Pharmacey, 30*(5), pp.591–595, (In Chinese).
14. Wang, X., Li, H.J., Yu, D.H., Sun, W.Y., Xu, H.M., He, Y.F. and Zhao, Q.C., **2019**. Rotundic acid induces Cas3-MCF-7 cell apoptosis through the p53 pathway. *Oncology Letters*, *17*(1), pp.630–637.
15. He, Y.F., Nan, M.L., Sun, J.M., Meng, Z.J., Yue, F.G., Zhao, Q.C., Yang, X.H. and Wang, H., **2012**. Synthesis, characterization and cytotoxicity of new rotundic acid derivatives. *Molecules, 17*(2), pp.1278–1291.
16. Muppidi, J., Porter, M. and Siegel, R.M., **2004**. Measurement of apoptosis and other forms of cell death. *Current Protocols in Immunology*, *59*(1), pp.3–17.
17. Wang, W., Liu, X., Guo, X. and Quan, H., **2018**. Mitofusin-2 triggers cervical carcinoma cell hela apoptosis via mitochondrial pathway in mouse model. *Cellular Physiology and Biochemistry*, *46*(1), pp.69–81.
18. Ando, K., Yokochi, T., Mukai, A., Wei, G., Li, Y., Kramer, S., Ozaki, T., Maehara, Y. and Nakagawara, A., **2019**. Tumor suppressor KIF1Bβ regulates mitochondrial apoptosis in collaboration with YME1L1. *Molecular Carcinogenesis*, *58*(7), pp.1134–1144.
19. Schwartz, G.K. and Shah, M.A., **2005**. Targeting the cell cycle: A new approach to cancer therapy. *Journal of Clinical Oncology*, *23*(36), pp.9408–9421.

20. Feng, J., Bian, T.T., Yuan, A. and Liu, Y.F., **2016**. The relationship of P21, CyclinD1 expression and the clinical pathology in squamous cell carcinoma of the penis. *Medical Journal of Communications*, *30*(6), pp.577–588, (in Chinese).
21. Park, H.J., Kim, M.J., Ha, E. and Chung, J.H., **2008**. Apoptotic effect of hesperidin through caspase3 activation in human colon cancer cells, SNU-C4. *Phytomedicine*, *15*(1–2), pp.147–151.
22. Shi, Y.R., Liu, J., He, W. and Yang, Y., **2016**. Expression of microRNA 218 in cervical cancer and its effect on proliferation, apoptosis and invasion of HeLa cells. *Journal of Sichuan University Medical Science Edition*, *47*(5), pp.697–702, (in Chinese with English abstract).
23. Wang, X., Yi, Y., Lv, Q., Zhang, J., Wu, K., Wu, W. and Zhang, W., **2018**. Novel 1, 3, 5-triazine derivatives exert potent anti-cervical cancer effects by modulating Bax, Bcl2 and Caspases expression. *Chemical Biology & Drug Design*, *91*(3), pp.728–734.
24. Adams, J.M. and Cory, S., **2007**. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene*, *26*(9), pp.1324–1337.
25. Wei, L., Qin, X.P., Zhao, X.X. and Wang, W., **2017**. Bcl-2 associated athanogene 3 affects the epithelial-mesenchymal transition in human cervical cancer. *Zhonghua Fu Chan Ke Za Zhi, 52*(8), pp.551–557, (In Chinese with English abstract).
26. Frenzel, A., Grespi, F., Chmelewskij, W. and Villunger, A., **2009**. Bcl2 family proteins in carcinogenesis and the treatment of cancer. *Apoptosis*, *14*(4), pp.584–596.
27. Sun, L.Y., Tao, J.Z., Yan, B. and Lin, J.S., **2017**. Inhibitory effects of FKBP14 on human cervical cancer cells. *Molecular Medicine Reports*, *16*(4), pp.4265–4272.
28. Dong, Y.J. and Gao, W.J., **2012**. The role and relationship of bcl-2, bax and caspase-3 in apoptosis. *Chinese Journal of Gerontology, 32*(21), pp.4828–4830.
29. Yang, B., Li, H., Ruan, Q.F., Xue, Y.Y., Cao, D., Zhou, X.H., Jiang, S.Q., Yi, T., Jin, J. and Zhao, Z.X., **2018**. A facile and selective approach to the qualitative and quantitative analysis of triterpenoids and phenylpropanoids by UPLC/Q-TOF-MS/MS for the quality control of Ilex rotunda. *Journal of Pharmaceutical and Biomedical Analysis*, *157*, pp.44–58.
30. Yang, B., Zhu, J.P., Rong, L., Jin, J., Cao, D., Li, H., Zhou, X.H. and Zhao, Z.X., **2018**. Triterpenoids with antiplatelet aggregation activity from Ilex rotunda. *Phytochemistry*, *145*, pp.179–186.
31. Liu, W.J., Peng, Y.Y., Chen, H., Liu, X.F., Liang, J.Y. and Sun, J.B., **2017**. Triterpenoid saponins with potential cytotoxic activities from the root bark of ilex rotunda thunb. *Chemistry & Biodiversity*, *14*(2), Article ID: e1600209.
32. Tan, T., Luo, Y., Zhong, C.C., Xu, X. and Feng, Y., **2017**. Comprehensive profiling and characterization of coumarins from roots, stems, leaves, branches, and seeds of chimonanthus nitensoliv. Using ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry combined with modified mass defect filter. *Journal of Pharmaceutical and Biomedical Analysis*, *141*, pp.140–148.
33. Cao, D., Wang, Q., Jin, J., Qiu, M., Zhou, L., Zhou, X., Li, H. and Zhao, Z., **2018**. Simultaneous qualitative and quantitative analyses of triterpenoids in ilex pubescens by ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *Phytochemical Analysis*, *29*(2), pp.168–179.
34. Wang, L.L., Kong, L., Liu, H., Zhang, Y., Zhang, L., Liu, X., Yuan, F., Li, Y. and Zuo, Z., **2019**. Design and synthesis of novel artemisinin derivatives with potent activities against colorectal cancer in vitro and in vivo. *European Journal of Medicinal Chemistry*, *182*, Article ID: 111665.
35. Chen, C.P., Chen, K., Feng, Z., Wen, X. and Sun, H., **2019**. Synergistic antitumor activity of artesunate and hdac inhibitors through elevating heme synthesis via synergistic upregulation of alas1 expression. *Acta Pharmaceutica Sinica B*, *9*(5), pp.937–951.
36. Bao, Y., Zhao, L., Wu, J., Jiang, S., Wang, Z. and Jin, Y., **2019**. Photo-induced synthesis of axinastatin 3 analogs, the secondary structures and their in vitro antitumor activities. *Bioorganic & Medicinal Chemistry Letters*, *29*(22), Article ID: 126730.

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