International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2022.1199.1209

Research Article
Ginsenoside Rk1 Induced Apoptosis in Ovarian Cancer SK-OV-3 Cells via ROS-Mediated Caspase Signaling Pathway

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Abstract

Background and Objective: Ovarian cancer has the highest mortality rate of all gynecologic cancers, exploring natural plant-derived antitumor drugs has become indispensable in ovarian cancer treatment. Ginsenoside Rk1 exerted numerous pharmacological activities including anti-tumour and anti-inflammation. The present study explored the effect of ginsenoside Rk1 (G-Rk1) on the proliferation and apoptosis in SK-OV-3 ovarian cancer cells and deepened its mechanism of action. **Materials and Methods:** Cell viability was measured by MTT assay. Changes in mitochondrial membrane potential (MMP) and Hoechst 33258 staining were used to detect cell apoptosis. Western blot was used to detect the release of cytochrome C and expression of apoptosis-related proteins Bcl-2, Bax and cleaved caspase-3, cleaved caspase-9. Additionally, flow cytometry was used to detect cellular cycles and apoptosis. **Results:** The G-Rk1 significantly inhibited cell viability dose-dependently ($p<0.01$). In addition, G-Rk1-induced apoptosis was investigated through reactive oxygen species (ROS) and mitochondrial membrane potential loss. It was observed that G-Rk1 can not only induce cell cycle arrest at the G1 phase but also inhibit the proliferation ability of SK-OV-3 cells. Importantly, western blot analysis confirmed that more cytochrome c was released from mitochondria resulting in caspase activation ($p<0.05$). **Conclusion:** Therefore, the findings from the present study exhibited that G-Rk1 could inhibit the proliferation of SK-OV-3 ovarian cancer cells and maybe as a candidate drug for anti-ovarian cancer soon.

Key words: Ginsenoside Rk1, ovarian cancer, SK-OV-3 cells, ROS, cell apoptosis, cell cycle, mitochondria

Citation: Fan, M.L., W.Y. Su, Y.B. Liu, J.N. Hu and J.T. Zhang *et al.*, 2022. Ginsenoside Rk1 induced apoptosis in ovarian cancer SK-OV-3 cells via ROS-mediated caspase signaling pathway. Int. J. Pharmacol., 18: 1199-1209.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ovarian cancer ranks sixth in cancer death among women (3% of all cancers among women), accounting for more deaths than any other cancer of the female reproductive system[1]. The most common type of ovarian cancer is epithelial ovarian carcinoma, which accounts for 85-90% of all ovarian cancers[2].

Ovarian cancer has the highest mortality rate of all gynecologic cancers because its early symptoms are nonspecific and most patients present an advanced stage of this disease during initial diagnosis[3]. Despite intensive multimodal therapy (chemotherapy and surgery), ovarian cancer still has a poor prognosis tumorigenesis and malignant transformation are caused by overexpression of the cell survival pathway and normal cellular senescence or apoptosis. Thus, manipulation of the cell survival pathway can reduce the malignant potential of these tumours and provide a pathway to develop new therapies[4]. Therefore, exploring natural plant-derived antitumor drugs with high efficiency and low toxicity has become a popular research topic in ovarian cancer treatment.

Natural compounds from herbs or medicinal plants are widely used for therapy and prevention of cancers[5,6]. Ginseng, the root of *Panax ginseng* C.A. Meyer (ginseng) and a famous traditional Chinese medicine (TCM), has been widely used in China, Korea and Japan due to its powerful pharmacological activity. Previous studies have also demonstrated that ginsenosides are the main active constituent in ginseng[7]. Ginsenosides in white ginseng underwent deglycosylation, dehydration and isomerization to generate many rare ginsenosides during the steaming process, of these rare ginsenosides, G-Rk1 (Fig. 1a) was the major one produced from protopanaxadiol-type ginsenosides such as Rb1, Rb2, Rb3, Rc and Rd during ginseng process[8]. It is well known to have various pharmacological activities including anti-cancer[9], anti-inflammatory[10] and anti-platelet aggregation[11]. Interestingly, ginsenoside Rg5, the isomer of G-Rk1, have been shown to inhibit the proliferation and promote apoptosis of ovarian cancer cells[12]. However, there are fewer reports about other saponins against ovarian cancer.

Some rare ginsenosides, including ginsenosides Rg3, Rh2, F2 and compound K, are more pharmacologically active than major ginsenosides such as ginsenoside Re, Rb1, Rb2 etc.[13-15]. Accumulating evidence has revealed that the major beneficial effects of ginsenoside have anti-inflammation, anti-diabetes and anti-tumour[16,17]. In particular, the rare ginsenosides are excellent in killing tumour and cancer cells. According to the literature we have, the research of ginsenosides on ovarian cancer is still relatively limited, especially the newly discovered rare saponins such as Rk1 and Rg5.

Based on the previous research results on the preparation and separation of rare ginsenosides, we first illustrated the effect of G-Rk1 on the proliferation and apoptosis of ovarian cancer (SK-OV-3 cell) and its underlying mechanism. We do not doubt that our results will provide the necessary theoretical basis for the in-depth development of ginsenoside on ovarian cancer and the development of anti-tumour drugs soon.

The present study explored the effect of ginsenoside Rk1 (G-Rk1) on the proliferation and apoptosis in SK-OV-3 ovarian cancer cells and deepened its mechanism of action.

MATERIALS AND METHODS

Reagents and chemicals: The G-Rk1 (purity >98%, HPLC analysis) were converted from crude saponins and purified by using the semi-preparative HPLC method in our lab[18]. Cisplatin (CDDP, purity $\geq$99.0%) was purchased from Sigma Chemicals (St. Louis, Missouri). Dulbecco's Modified Eagle's Medium (DMEM) and penicillin-streptomycin solution were obtained from HyClone Laboratories (Logan, Utah). Fetal Bovine Serum (FBS) was purchased from CLARK Bioscience (Richmond, Virginia). The ROS dye kit was purchased from Wanlei Biotechnology (Shenyang China). Hoechst 33258 dye kit was obtained from Beyotime Co., Ltd. (Shanghai, China). A mitochondrial membrane potential detection kit was purchased from Nanjing Jiancheng Biological Research Institute (Nanjing, China). Cell Cycle and Apoptosis Analysis Kit were purchased from Beyotime Co., Ltd. (Shanghai, China). The antibodies for Bax, Bcl-2, Cytochrome, caspase-3, cleaved caspase-3, caspase-9, cleaved caspase-9, β-actin and the horseradish peroxidase-conjugated antirabbit antibodies were procured from Cell Signaling Technology (Danvers, Massachusetts). All other reagents and chemicals, unless indicated, were obtained from Beijing Chemical Factory (Beijing, China). The study was carried out at the College of Chinese Medicinal Materials, Jilin Agricultural University from November, 2019 to September, 2020.

Cell culture and viability assay: The SK-OV-3 cells were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO_2 and were sub-cultured every 2-3 days.

The cells were seeded in 96-well plates to reach a density of 510^4 cells/well and incubated overnight. When the cells reached 60% confluence, G-Rk1 were added to make concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μM, respectively. Similarly, we also evaluated the toxicity effect of cisplatin on SK-OV-3 cells. Cells were treated with cisplatin at different concentrations (1, 2, 4, 8, 16, 32, 64, 128 and 256 μM) dividedly before MTT determination. After incubation for 24 h, the cell viability was measured at 490 nm by a microplate reader (Nano, Germany).

ROS staining: The SK-OV-3 cells were cultured in 6-well microplates and treated with 1 μM DCFH-DA at 37°C for 30 min. The medium was removed after staining and cells were washed by PBS two times before imaging. Cisplatin (32 μM) was used as positive control and relative ROS fluorescence intensity of treated cells was expressed as a percentage of the cisplatin-induced group (Leica TCS SP8, Solms, Germany).

Hoechst 33258 staining: Nuclear morphological changes of SK-OV-3 cells were analyzed by Hoechst 33258 staining[19]. Briefly, after co-incubation with G-Rk1 and cisplatin for 24 hrs, SK-OV-3 cells were fixed in stationary liquid overnight and stained by Hoechst 33258 solution (10 μg mL^{-1}). After washing with PBS (0.01 M, pH 7.4) three times, stained nuclei were visualized under UV light and photographed by a fluorescent microscope (Leica TCS SP8, Germany). The result was quantified by Image-Pro Plus 6.0 software.

Immunofluorescence staining: The SK-OV-3 cells were cultured in 6-well microplates and then treated with PBS (0.01 M, pH 7.4) three times. After being washed with PBS three times, the SK-OV-3 cells were incubated with 4% paraformaldehyde fix solution for 15 min, then blocking and permeabilization was performed with 0.2% Triton-X100 in PBS containing 5% bovine serum albumin for 15 min at room temperature. Then, the cell was incubated with primary antibodies including mouse anti-Bax (1:200) at 4°C overnight. After three times washing with PBS, the cells were incubated with SABC-Cy3 (BOSTER, Wuhan, China) for 30 min at 37°C, then were subject to 4, 6 diamidino-2-phenylindole (DAPI) staining. Finally, the stained cells were observed under a fluorescence microscope (Leica TCS SP8, Germany) and the intensity of immunofluorescence was analyzed using Image-Pro Plus 6.0 software.

Mitochondrial membrane potential detection (MMP): The MMP was examined by staining the cells with the fluorescent probe JC-1[12] (Beyotime, Shanghai). Briefly, after treatments, the cells were collected, resuspended in 500 μL of PBS and incubated in 0.5 mL JC-1 working solution in darkness at 37°C for 20 min. Then the stained cells were analyzed by fluorescent microscope (Leica TCS SP8, Germany).

Cell cycle and apoptosis analysis: Cell cycle progression was evaluated using the Cell Cycle and Apoptosis Analysis Kit (Beyotime Co., Ltd. Shanghai, China). In brief, after the treatments, the cells were collected, stained with propidium iodide (PI) for 30 min in the dark. Then the stained cells were analyzed by flow cytometry.

Cell apoptosis was evaluated using the Annexin V-FITC apoptosis analysis kit (Tianjin Sungene Biotech Co., Ltd.). Briefly, after the treatments, the cells were collected, washed with PBS and suspended in 500 μL binding buffer with 5 μL of Annexin V-FITC and 5 μL PI for 5 min in the dark. Then the stained cells were analyzed by flow cytometry.

Western blot analysis: Total protein was extracted using RIPA buffer (1:10, g/v) and the protein concentrations were measured by BCA protein assay kit (Thermo Scientific, Waltham, Massachusetts)[20]. The protein, 5 mg mL^{-1}, were loaded on 12% SDS-PAGE gel (50 μg per lane) and transferred to the polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in tris-buffered saline (TBS) containing 0.1% Tween-20 for at least 1 hr and incubated overnight with primary antibodies including Bax (1:2000), Bcl-2 (1:2000), Cytochrome C (1:1000), caspase-3 (1:1000), caspase-9 (1:1000), cleaved caspase-3 (1:1000), cleaved caspase-9, β-actin (1:2000) at 4°C and then incubated with secondary antibodies for 1 hr at room temperature after washing with TBS-T. The ECL substrate (Thermo Scientific, Massachusetts) was used for signal detection.

Statistical analysis: Data are expressed as Means±Standard Deviation (Mean±SD). The student's t-test was employed for comparisons between two groups. Multiple comparisons were performed using one-way ANOVA, $p<0.05$ were considered significant. Analysis was performed using GraphPad Prism 8.0.2. (GraphPad Software, La Jolla, CA, USA).

RESULTS

G-Rk1 on cell proliferation and cycle progression: The effect of G-Rk1 on SK-OV-3 cell viability was examined by MTT assay. The cell viability of the normal group was defined as 100% and other groups were expressed as a percentage of the normal group. As shown in Fig. 1b-c, compared with the normal group (Normal, N), with the treatment of G-Rk1 and cisplatin, the survival rate of cells gradually decreased with

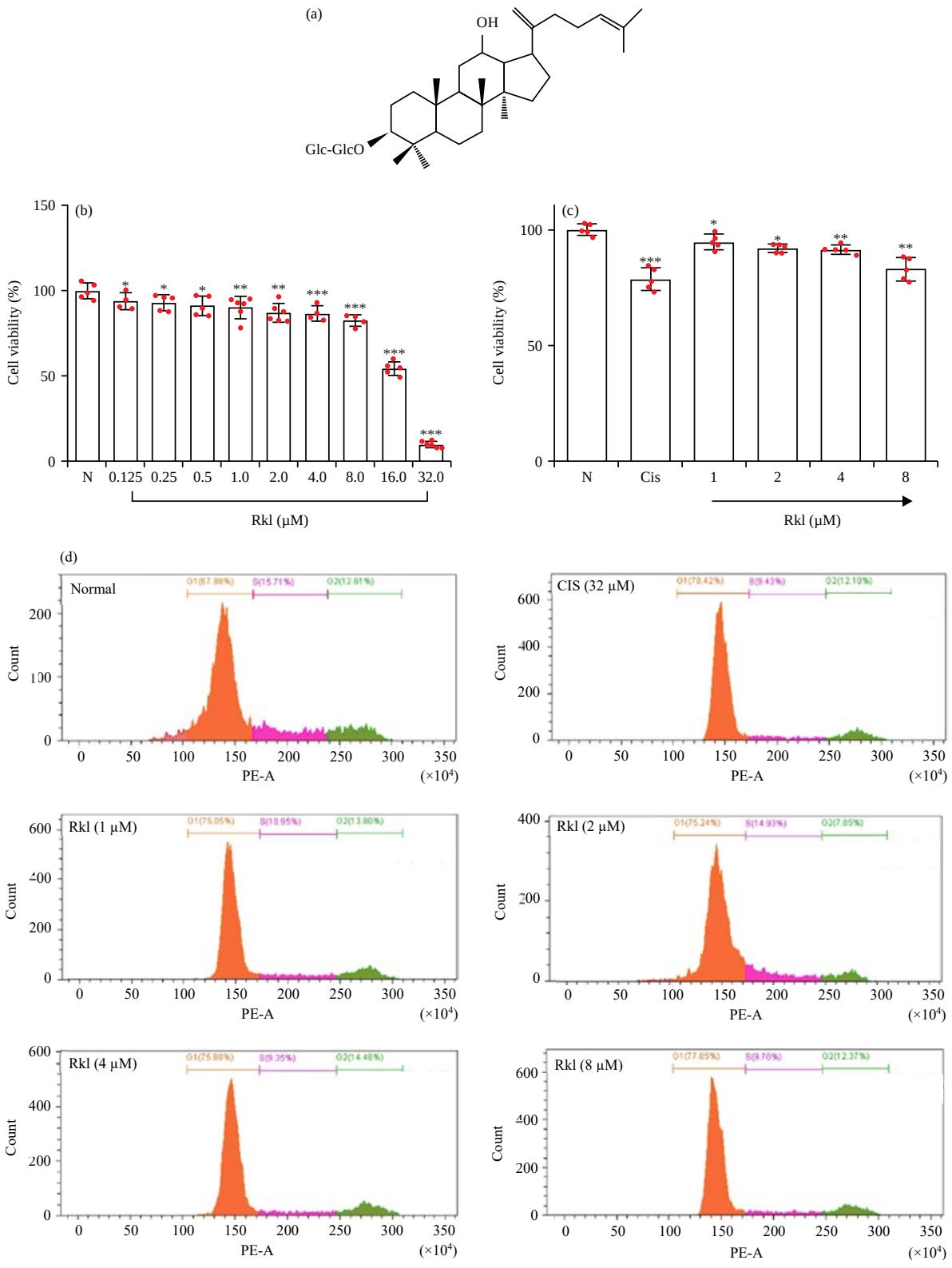

Fig. 1(a-d): Effects of G-Rk1 on SK-OV-3 cell, (a) Chemical structures of G-Rk1, (b) Effect of G-Rk1 on cells viability, (c) Effect of G-Rk1 and cisplatin on cells viability and (d) Cell cycle progression was analyzed by flow cytometry

Data are expressed as Mean±SD (n≥6), *p<0.05, **p<0.01 and ***p<0.0001 vs. normal group

dose-dependent relationship, with a significant dose-dependent and relative in comparison, G-Rk1 has a stronger inhibitory effect on SK-OV-3 cell viability than cisplatin. The IC_{50} value of G-Rk1 on SK-OV-3 cells for 24 hrs was 23.242 μM. Therefore, the concentration of 1, 2, 4 and 8 μM were selected in subsequent experiments and 32 μM of cisplatin was used as the subsequent experimental concentration.

To further investigate the effect of G-Rk1, cell cycle assays were performed for SK-OV-3 cells. G-Rk1 regulates G1/S cell cycle arrest, the G2-phase and S-phase were reduced compared with normal groups, while the G1-phase was increased (Fig. 1d). In the normal cells, the proportions of G1, S and G2 phases were 67.98, 15.71 and 12.61%. After incubating with 8 μM G-Rk1, the proportions of G1, S and G2 phases were 77.85, 9.70 and 12.37%, respectively ($p<0.01$). These results showed that the percentage of S and G2/M phases cells decreased, while those in the G1 phase increased after treatment with G-Rk1 ($p<0.05$ and $p<0.01$).

G-Rk1 inhibits the ROS overproduction and cell apoptosis: As shown in Fig. 2a, G-Rk1 and cisplatin-treated cells showed typical apoptotic morphology, whereas, cells not treated with G-Rk1 and cisplatin had normal morphology. We further

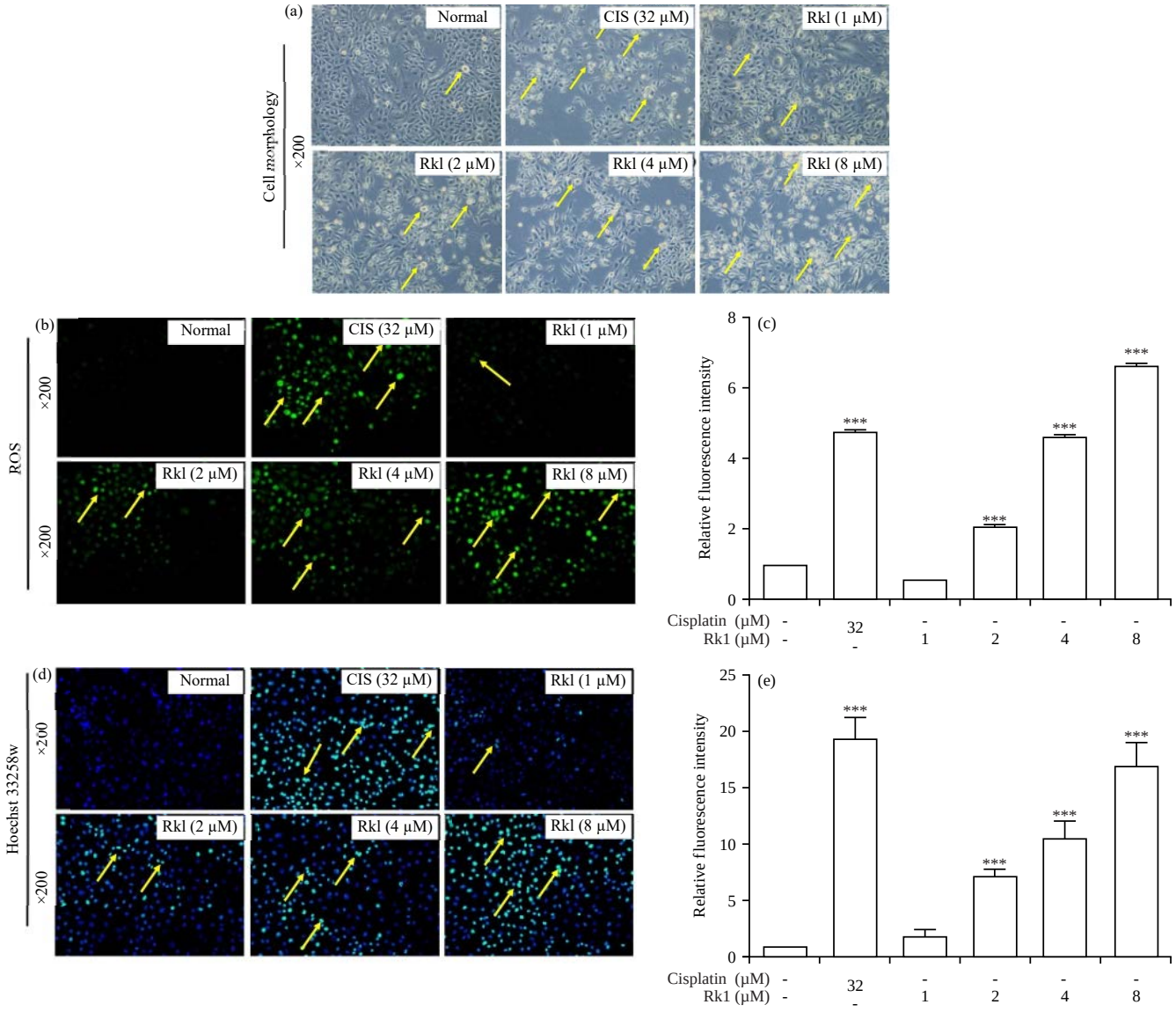

Fig. 2(a-e): Effects of G-Rk1 on apoptosis in SK-OV-3 cell, (a) Cell morphology of SK-OV-3 cells, (b) ROS staining of SK-OV-3 cells (200×), (c) Fluorescence intensity of ROS staining, (d) Hoechst 33258 staining of SK-OV-3 cells (200×) and (e) Fluorescence intensities of the Hoechst 33258 staining

Data are expressed as Mean±SD, *p<0.05, **p<0.01 and ***p<0.0001 vs. normal group

verified apoptosis by ROS stating (Fig. 2b-c), after cisplatin treatment, intracellular ROS generation was significantly increased. The G-Rk1 treatment for 24 hrs also significantly increased the intracellular ROS levels as the concentration of G-Rk1 increased ($p<0.05$).

According to the results of Hoechst 33258 staining in Fig. 2d, e, the nucleus of the normal group was morphologically intact and neatly arranged with a clear outline along with evenly and slightly stained chromatin. In the cisplatin group, a large proportion and high density of blue apoptosis of cells and small chunks of nucleus distributed clearly, indicating severe apoptosis after cisplatin challenge. The G-Rk1 treatment also significantly increased apoptosis as the concentration of G-Rk1 increased.

G-Rk1 regulates the expression of Bcl-2 family proteins: The Bcl-2 family proteins are related proteins regulating apoptosis. Bcl-2 is an anti-apoptotic protein and a member of the Bcl-2 family of the apoptosis regulator proteins. To further evaluate the effect of G-Rk1, we examined the effect of G-Rk1 on Bax expression in SK-OV-3 cell using immunofluorescence staining and western blot. The experiment is illustrated in Fig. 3a, b, Bax-positive puncta were rarely observed in the normal group ($p<0.05$, $p<0.01$). Concomitant with the immunofluorescence staining of Bax, western blot analysis of the Bcl-2 family proteins showed that the protein expression of Bax was increased in the SK-OV-3 cell. In contrast, a significant decrease of Bcl-2 was found in the G-Rk1 treatment groups compared to the normal group ($p<0.01$ and $p<0.05$) (Fig. 3c-e).

G-Rk1 reduces the mitochondrial membrane potential: To detect the change of G-Rk1-induced membrane potential in mitochondria, the sensitive fluorescent probes JC-1 was used as indicators of mitochondrial dysfunction. As shown in Fig. 4, the normal cells showed red fluorescence (JC-1 aggregates), indicating high membrane potential. The SK-OV-3 cells treated with G-Rk1 at a concentration of 1, 2, 4 and 8 μM for 24 hrs showed less red but increased green fluorescence, which means that membrane potential is low. These results suggest that G-Rk1 could induce apoptosis in SK-OV-3 cells by promoting mitochondrial dysfunction.

G-Rk1 regulates cell apoptosis and caspase activation: To determine the effect of caspases on the apoptotic process, we investigated whether the mechanism of the G-Rk1-induced cell death employed caspase proteins by examining their expression according to western blot analysis. From the results, we found increased levels of cleaved caspase 3 and caspase 9 with G-Rk1-pretreatment by western blot analysis (Fig. 5a-d).

In addition, we analyzed the apoptosis rate of G-Rk1-treated SK-OV-3 cells by flow cytometry following Annexin V-FITC/PI double staining. Early and late apoptotic cell populations were shown as Annexin V+/PI-and Annexin V+/PI+ cells, respectively and both were considered apoptotic. G-Rk1 treatment with various concentrations (1, 2, 4 and 8 μM) significantly increased the percentage of apoptotic cells (1.84, 2.18, 33.65 and 51.28%, respectively) in a dose-dependent manner (Fig. 5e).

DISCUSSION

Ovarian, cervical and endometrial cancers are common gynaecological malignancies, whose incidence are increasing[21-23]. Furthermore, ovarian cancer is the highest among the gynaecological malignant tumours. The primary treatment approaches for ovarian in clinical practice are surgical resection, chemotherapy and radiotherapy, however, efficacy can be poor due to high rates of recurrence and a poor prognosis. Therefore, the prevention of ovarian is essential. In this study, G-Rk1 induces apoptosis in SK-OV-3 cells via the ROS-mediated mitochondrial pathway.

As known, ROS could induce apoptosis through activation of mitochondrial-mediated intrinsic apoptotic pathway[22]. At the same time, the mitochondrion is a major site for ROS production and excessive ROS accumulation can further damage mitochondria [24-26]. In the present study, we found that G-Rk1 significantly increased the production of intracellular and mitochondrial ROS (Fig. 2b) and a significant progressive decline in MMP (Fig. 4). However, Numerous studies have revealed that high intracellular levels of ROS not only result in mitochondrial DNA damage and release of cytochrome c from mitochondria to cytosol, thereby triggering caspase-dependent or caspase-independent apoptosis[27,28]. Likewise, ROS has good antitumor effects and can inhibit the proliferation and survival of various tumour cells by inducing cell cycle arrest and apoptosis [29]. Inducing cell cycle arrest is an effective method to suppress cancer cell growth[30]. Various anticancer agents trigger cell cycle arrest[31]. Similarly, ginsenosides can induce cell cycle arrest[32-34]. Ginsenoside Rh2 arrests G0/G1 phase in A549 human lung adenocarcinoma cells through the reduction of cyclin D1 and CDK4[32]. The results were similar to these conclusions, we demonstrated that Rk1 triggered G1 phase arrest in SK-OV-3 cells (Fig. 1d).

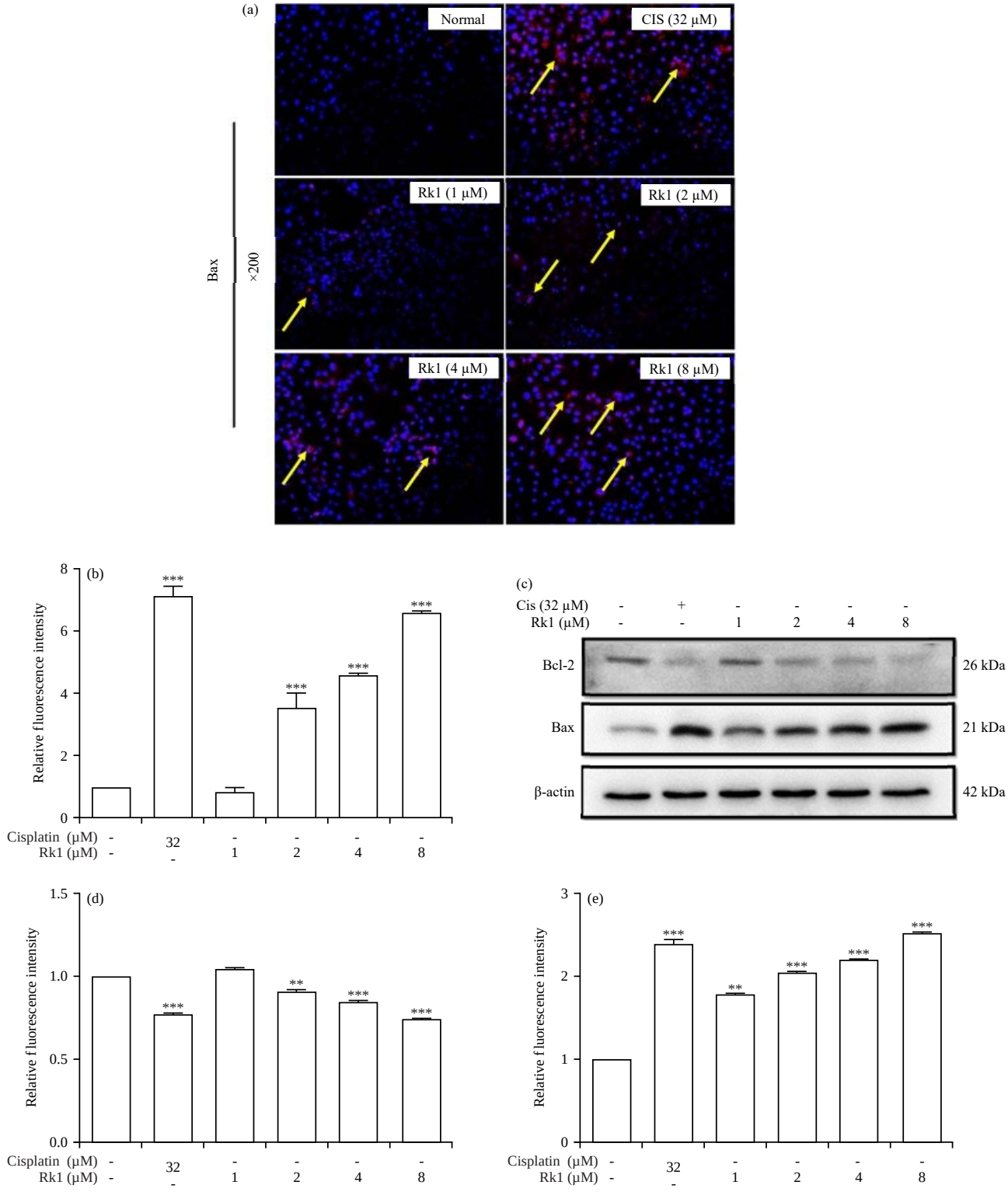

Fig. 3(a-e): G-Rk1 regulates the expression of Bcl-2 family proteins in SK-OV-3 cell, (a) Immunofluorescent image of the expression levels of Bax (red) in SK-OV-3 cell expression, (b) Effects of G-Rk1 on the protein expressions of Bax and Bcl-2, (c) Western blot analysis of Bax and Bcl-2 and (d-e) Quantification of Bax and Bcl-2 protein expression were performed by densitometric analysis

Data are expressed as Mean±SD, *p<0.05, **p<0.01 and ***p<0.0001 vs. normal group

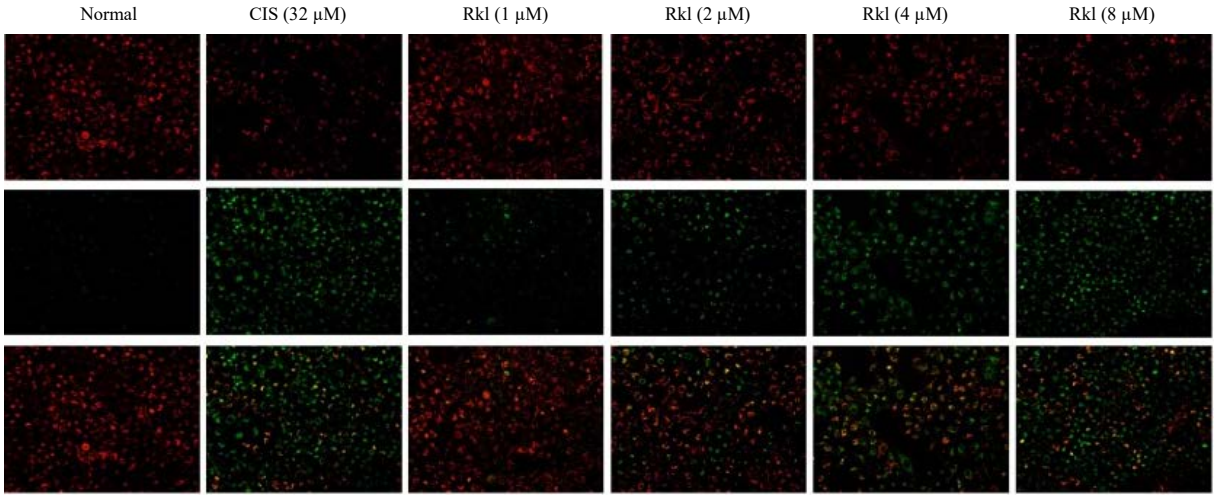

Fig. 4: G-Rk1 reduces the mitochondrial membrane potential
Mitochondrial membrane potential (JC-1) staining of SK-OV-3 cell (200×)

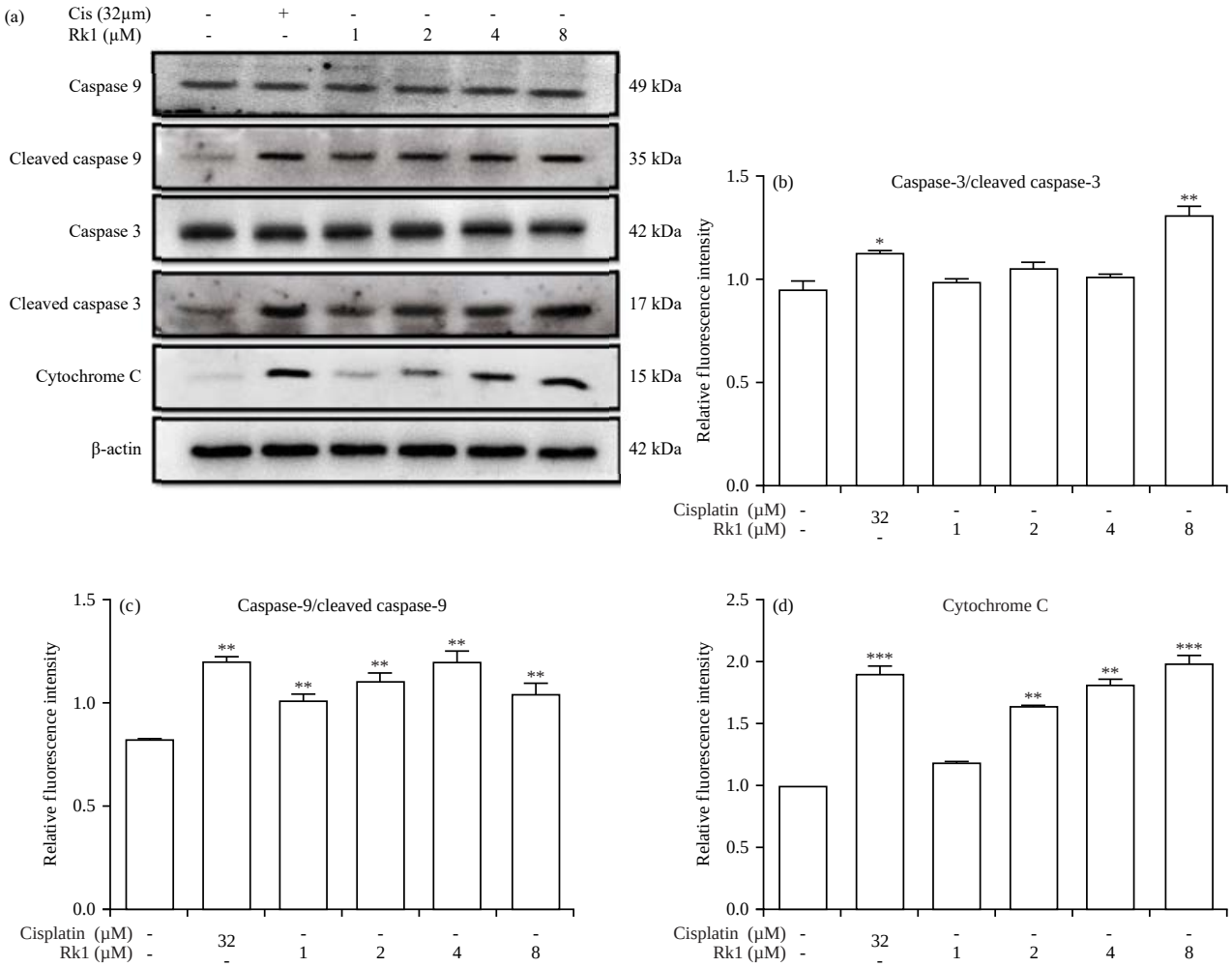

Fig. 5(a-e): Continue

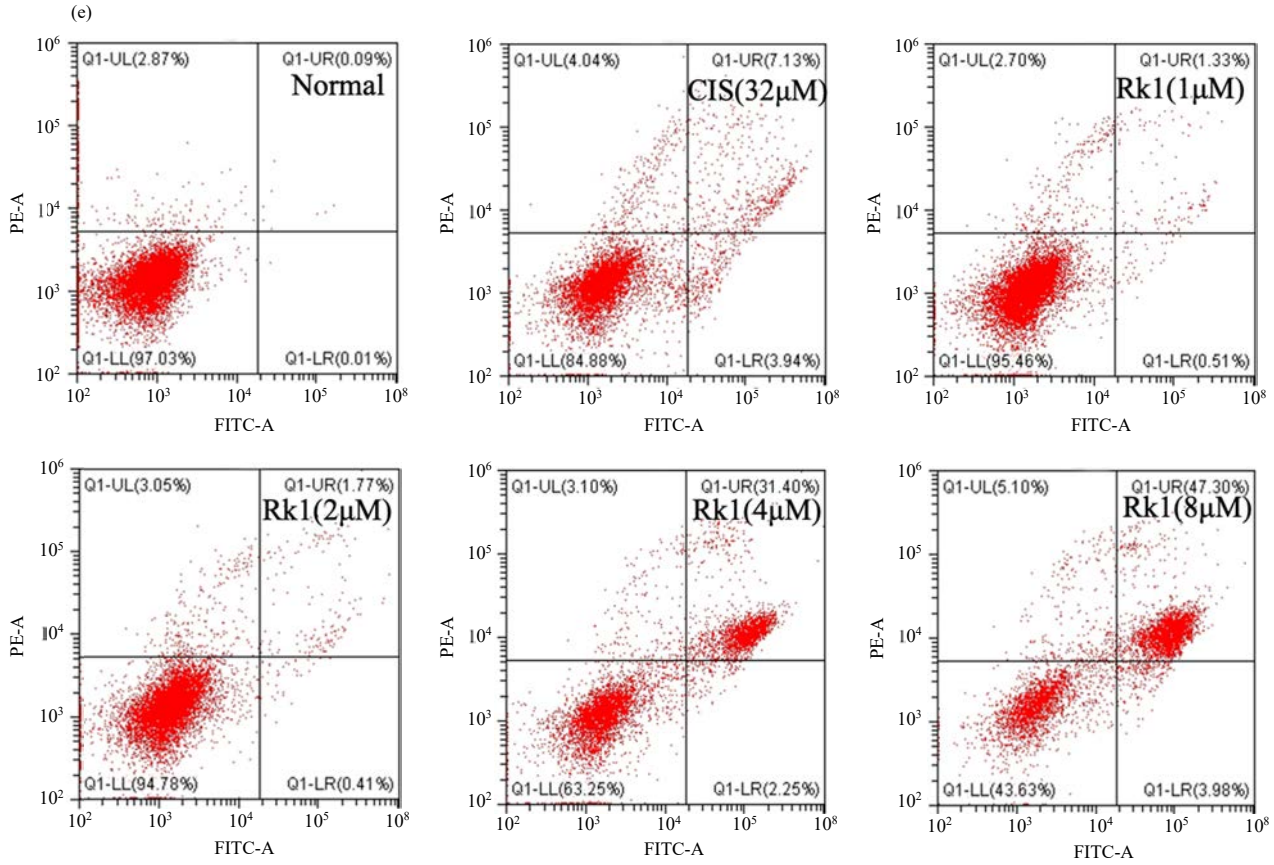

Fig. 5(a-e): G-Rk1 Regulates apoptosis and caspase activation in SK-OV-3 Cells, (a) Effects of G-Rk1 on the protein expressions of cleaved caspase-3, caspase-3, cleaved caspase-9 and caspase-9, Cytochrome C, (b-d) Quantification of relative protein contents were performed by densitometric analysis and (e) Cell apoptosis was analysed by flow cytometry
Data are expressed as Mean±SD, *p<0.05, **p<0.01, ***p<0.0001 vs. normal group

Cell cycle arrest is closely linked to apoptosis[35]. Cell proliferation is regulated by cell cycle progression, while apoptosis is related to cell cycle arrest[36]. Agents that induce cell cycle arrest and apoptosis could be potential therapeutic drugs for cancer therapy. Indeed, the mitochondrial/caspase-mediated signalling cascade is an important pathway of apoptosis characterized by mitochondrial membrane permeabilization[37]. The Bcl-2 and Bax serve important roles in regulating caspase-dependent and caspase-independent apoptosis, mediated by the mitochondrial pathway[38]. Furthermore, the mitochondrial membrane permeability may be increased through Bax activation[39]. Western blot and Immunofluorescence analysis demonstrated that G-Rk1-treatment markedly increased the protein level of Bax (Fig. 3). Moreover, Bax, a pro-apoptotic protein, can translocate from the cytoplasm to the mitochondria and inserts into the mitochondrial membrane, which induces an increase in mitochondrial membrane permeability and results in the release of cytochrome c[40]. This study further demonstrated that G-Rk1 promoted the expression levels of Bax and cleaved caspase 9, cleaved caspase 3 and reduced the expression of Bcl-2 ($p<0.01$). Furthermore, Rk1 caused cytochrome c release from mitochondria to cytosol. These findings demonstrated that Rk1 triggered apoptosis via mitochondrial pathways in SK-OV-3 cells.

CONCLUSION

Collectively, we found that G-Rk1 can induce cell cycle arrest and apoptosis in SK-OV-3 cells. Our results clarified that G-Rk1 suppressed the cell viability and induced G1 phase arrest and cellular apoptosis. G-Rk1 induce apoptotic cells death through mitochondria- and caspase-dependent pathways in SK-OV-3 cells. These results suggest that G-Rk1 can be a safe cancer therapeutic drug because it induces apoptosis in cancer cells and reduces migration ability through the enhancement of ROS signalling, even at low concentrations. However, studies on the efficacy and safety of humans are required to investigate the therapeutic potential of G-Rk1 for cancer.

SIGNIFICANCE STATEMENT

This study discovers that G-Rh1 can inhibit cell proliferation in SK-OV-3 cells that can be beneficial for the treatment of ovarian cancer. This study will help the researcher to explore an efficacy and safety method in the critical areas of cancer therapeutics.

ACKNOWLEDGMENTS

This study was supported by the grants of the National Natural Science Foundation of China (No. 82104465) and the Jilin Science and Technology Development Plan (No. 20200301037RQ).

REFERENCES

1. Kowsarnia, S. and N. Javadi, 2021. Ovarian cancer with breast metastasis and two pathogenic variants of BRCA1 gene. Cureus, Vol. 13. 10.7759/cureus.18691.
2. Ramalingam, P., 2016. Morphologic, immunophenotypic, and molecular features of epithelial ovarian cancer. Oncology (Williston Park), 30: 166-176.
3. Erturk, A., O. Kuru, U. Akgor, G. Boyraz and C. Cakir *et al*., 2020. Cytoreductive surgery including distal pancreatectomy with splenectomy in advanced stage ovarian cancer: Two centers analysis. Taiwanese J. Obstet. Gynecol., 59: 862-864.
4. Oh, J.M., J. Lee, W.T. Im and S. Chun, 2019. Ginsenoside Rk1 induces apoptosis in neuroblastoma cells through loss of mitochondrial membrane potential and activation of caspases. Int. J. Mol. Sci., Vol. 20. 10.3390/ijms20051213.
5. Li, Y.Z., S. Ren, X.T. Yan, H.P. Li and W. Li *et al*., 2018. Improvement of Cisplatin-induced renal dysfunction by *Schisandra chinensis* stems via anti-inflammation and anti-apoptosis effects. J. Ethnopharmacol., 217: 228-237.
6. Zhang, W., J. Hou, X. Yan, J. Leng and R. Li *et al*., 2018. *Platycodon grandiflorum* saponins ameliorate cisplatin-induced acute nephrotoxicity through the NF-κB-mediated inflammation and PI3K/Akt/apoptosis signaling pathways. Nutrients, Vol. 10. 10.3390/nu10091328.
7. Ren, S., J. Leng, X.Y. Xu, S. Jiang and Y.P. Wang *et al*., 2019. Ginsenoside Rb1, a major saponin from *Panax ginseng*, exerts protective effects against acetaminophen-induced hepatotoxicity in mice. Am. J. Chin. Med., 47: 1815-1831.
8. Kim, W.Y., J.M. Kim, S.B. Han, S.K. Lee and N.D. Kim *et al*., 2000. Steaming of ginseng at high temperature enhances biological activity. J. Nat. Prod., 63: 1702-1704.
9. Ko, H., Y.J. Kim, J.S Park, J.H. Park and H.O. Yang, 2009. Autophagy inhibition enhances apoptosis induced by ginsenoside Rk1 in hepatocellular carcinoma cells. Biosci. Biotechnol. Biochem., 73: 2183-2189.
10. Hu, J.N., X.Y. Xu, W. Li, Y.M. Wang, Y. Liu, Z. Wang and Y.P. Wang, 2019. Ginsenoside Rk1 ameliorates paracetamol-induced hepatotoxicity in mice through inhibition of inflammation, oxidative stress, nitrative stress and apoptosis. J. Ginseng Res., 43: 10-19.
11. Ju, H.K., J.G. Lee, M.K. Park, S.J. Park, C.H Lee, J.H Park and S.W. Kwon, 2012. Metabolomic investigation of the anti-platelet aggregation activity of ginsenoside Rk_1 reveals attenuated 12-HETE production. J. Proteome Res., 11: 4939-4946.
12. Liang, L.D., T. He, T.W. Du, Y.G. Fan, D.S Chen and Y. Wang, 2015. Ginsenoside-Rg5 induces apoptosis and DNA damage in human cervical cancer cells. Mol. Med. Rep., 11: 940-946.
13. Kim, J.K., C.H. Cui, M.H. Yoon, S.C. Kim and W.T. Im, 2012. Bioconversion of major ginsenosides Rg_1 to minor ginsenoside F_1 using novel recombinant ginsenoside hydrolyzing glycosidase cloned from *Sanguibacter keddieii* and enzyme characterization. J. Biotechnol., 161: 294-301.
14. Liu, C., K. Zuo, H. Yu, C. Sun and T. Zhang *et al*., 2015. Preparation of minor ginsenosides C-Mx and C-K from notoginseng leaf ginsenosides by a special ginsenosidase type-I. Process Biochem., 50: 2158-2167.
15. Liu, W., Z. Wang, J. Leng, H. Wei and S. Ren *et al*., 2020. 20(R)-ginsenoside Rg3, a product of high-efficiency thermal deglycosylation of ginsenoside Rd, exerts protective effects against scrotal heat-induced spermatogenic damage in mice. Biocell, 44: 655-669.
16. Li, W., M. Zhang, J. Gu, Z.J. Meng and L.C. Zhao *et al*., 2012. Hypoglycemic effect of protopanaxadiol-type ginsenosides and compound K on Type 2 diabetes mice induced by high-fat diet combining with streptozotocin via suppression of hepatic gluconeogenesis. Fitoterapia, 83: 192-198.
17. Kim, D.Y., H.D. Yuan, I.K. Chung and S.H. Chung, 2009. Compound K, intestinal metabolite of ginsenoside, attenuates hepatic lipid accumulation via AMPK activation in human hepatoma cells. J. Agric. Food Chem., 57: 1532-1537.
18. Hu, J.N., X.Y. Xu, S. Jiang, Y. Liu and Z. Liu *et al*., 2020. Protective effect of ginsenoside Rk1, a major rare saponin from black ginseng, on cisplatin-induced nephrotoxicity in HEK-293 cells. Kaohsiung J. Med. Sci., 36: 732-740.
19. Xing, J.J., J.G. Hou, Z.N. Ma, Z. Wang and S. Ren *et al*., 2019. Ginsenoside Rb3 provides protective effects against cisplatin-induced nephrotoxicity via regulation of AMPK-/mTOR-mediated autophagy and inhibition of apoptosis *in vitro* and *in vivo*. Cell Proliferation, Vol. 52. 10.1111/cpr.12627.
20. Liu, X., X. Mi, Z. Wang, M. Zhang and J. Hou *et al*., 2020. Ginsenoside Rg3 promotes regression from hepatic fibrosis through reducing inflammation-mediated autophagy signaling pathway. Cell Death Dis., Vol. 11. 10.1038/s41419-020-2597-7.

21. Dietl, J., 2014. Revisiting the pathogenesis of ovarian cancer: The central role of the fallopian tube. Arch. Gynecol. Obstet., 289: 241-246.
22. Acharya, U.R., S.V. Sree, L. Saba, F. Molinari, S. Guerriero and J.S. Suri, 2013. Ovarian tumor characterization and classification using ultrasound-A new online paradigm. J. Digit. Imaging, 26: 544-553.
23. Cormio, A., G. Cormio, C. Musicco, A.M. Sardanelli, G. Gasparre and M.N. Gadaleta, 2014. Mitochondrial changes in endometrial carcinoma: Possible role in tumor diagnosis and prognosis (Review). Oncol. Rep., 33: 1011-1018.
24. Cozzolino, M., A. Ferri, C. Valle and M.T. Carrì, 2013. Mitochondria and ALS: Implications from novel genes and pathways. Mol. Cell. Neurosci., 55: 44-49.
25. Perier, C., K. Tieu, C. Guégan, C. Caspersen and V. Jackson-Lewis *et al*., 2005. Complex I deficiency primes bax-dependent neuronal apoptosis through mitochondrial oxidative damage. Proc. Natl. Acad. Sci. USA, 102: 19126-19131.
26. Dunn, J.D, L.A. Alvarez, X. Zhang and T. Soldati, 2015. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. Redox Biol., 6: 472-485.
27. Bhardwaj, M., N.H. Kim, S. Paul, R. Jakhar, J. Han and S.C. Kang, 2016. 5-hydroxy-7-methoxyflavone triggers mitochondrial-associated cell death via reactive oxygen species signaling in human colon carcinoma cells. PLoS ONE, Vol. 11. 10.1371/journal.pone.0154525.
28. Mathew, J., L. Galarneau, A. Loranger, S. Gilbert and N. Marceau, 2008. Keratin–protein kinase C interaction in reactive oxygen species-induced hepatic cell death through mitochondrial signaling. Free Radical Biol. Med., 45: 413-424.
29. Zeferino R.C., N.S.R.S. Mota, V.M.A.S. Grinevicius, K.B. Filipe and P.M. Sulis *et al*., 2020. Targeting ROS overgeneration by N-benzyl-2-nitro-1-imidazole-acetamide as a potential therapeutic reposition approach for cancer therapy. Invest. New Drugs, 38: 785-799.
30. Kim, S.J. and A.K. Kim, 2014. Anti-breast cancer activity of fine black ginseng (*Panax ginseng* Meyer) and ginsenoside Rg5. J. Ginseng Res., 39: 125-134.
31. Shangguan, W.J., H. Li and Y.H. Zhang, 2013. Induction of G2/M phase cell cycle arrest and apoptosis by ginsenoside Rf in human osteosarcoma MG-63 cells through the mitochondrial pathway. Oncol. Rep., 31: 305-313.
32. Liu, X., Y. Sun, L. Yue, S. Li and X. Qi *et al*., 2016. JNK pathway and relative transcriptional factor were involved in ginsenoside Rh2-mediated G1 growth arrest and apoptosis in human lung adenocarcinoma A549 cells. Genet. Mol. Res., Vol. 15. 10.4238/gmr.15039003.
33. Wang, W., E.R. Rayburn, J. Hang, Y. Zhao, H. Wang and R. Zhang, 2009. Anti-lung cancer effects of novel ginsenoside 25-OCH_3-PPD. Lung Cancer, 65: 306-311.
34. Wu, Q., J. Deng, D. Fan, Z. Duan, C. Zhu, R. Fu and S. Wang, 2018. Ginsenoside Rh4 induces apoptosis and autophagic cell death through activation of the ROS/JNK/p53 pathway in colorectal cancer cells. Biochem. Pharmacol., 148: 64-74.
35. Zhang, C.M., J. Zhao and H.Y. Deng , 2013. MiR-155 promotes proliferation of human breast cancer MCF-7 cells through targeting tumor protein 53-induced nuclear protein 1. J. Biomed. Sci., Vol. 20. 10.1186/1423-0127-20-79.
36. Vermeulen, K., Z.N. Berneman and D.R. Van Bockstaele, 2003. Cell cycle and apoptosis. Cell Proliferation, 36: 165-175.
37. Hseu, Y.C., R.W. Lin, Y.C. Shen, K.Y. Lin and J.W. Liao *et al*., 2020. Flavokawain B and doxorubicin work synergistically to impede the propagation of gastric cancer cells via ROS-mediated apoptosis and autophagy pathways. Cancers, Vol. 12. 10.3390/cancers12092475.
38. Wang, M., K. Li, Z. Zou, L. Li and L. Zhu *et al*., 2018. Piperidine nitroxide tempol enhances cisplatin-induced apoptosis in ovarian cancer cells. Oncol. Lett., 16: 4847-4854.
39. Li, X., Y. Liu, S. Liao, C. Lin and A. Moro *et al*., 2021. Polyphyllin VII induces apoptosis and autophagy via mediating H_2O_2 levels and the JNK pathway in human osteosarcoma U2OS cells. Oncol. Rep., 45: 180-190.
40. Jia, J., S. Dai, X. Sun, Y. Sang and Z. Xu *et al*., 2015. A preliminary study of the effect of ECRG4 overexpression on the proliferation and apoptosis of human laryngeal cancer cells and the underlying mechanisms Mol. Med. Rep., 12: 5058-5064.