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Peptides derived from Royal Jelly induce gastric cancer cells apoptosis by cell cycle arresting

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Abstract

Functions of Royal jelly polysaccharide have been widely explored, yet, the anti-tumor potency of Royal jelly peptides (RJPs) has never been explored. However, the anti-tumor property of RJPs was rarely explored. The present study evaluated the *in vitro* effects of RJPs on human gastric cancer cell lines. We found that RJP-1 (SHGEWL) significantly inhibited the proliferation of the MKN-45 cells in a dose- and time-dependent manner. Further study revealed that RJP-1 regulated the p53/MDM2 state and blocked the cell cycle in the G0/G1 phase, which accompanied with inhibiting the CDK2, 4 and cyclin D, E expressions. In addition, RJP-1 significantly increased the Bax/Bcl-2 ratio and activated the Caspases, thereby inducing the MKN-45 cell apoptosis. The current findings reveal that the bioactive peptides in the Royal jelly maybe shining anti-cancer agents.

Keywords: Royal jelly; Gastric cancer; Cell cycle arrest, Apoptosis

1. Introduction

There exist almost one million new cases of gastric cancer (GC) every year worldwide and half of these occur in Eastern Asia, China in particular. Although the incidence of GC has declined over the years, it remains the fifth most common cancer and the third leading cause of cancer-related death in the world. The poor prognosis of GC is mainly attributed to tumor metastasis and recurrence[1]. Peritoneal metastasis is the most frequent dissemination pattern and accounts for over 40 percent among all recurrence cases. Once peritoneal recurrence occurs, the mean survival time is only 1.5 years[2]. Gastric cancer is one of the most common malignant tumors in China, with the high-risk age of the disease is above 50 years old. In the past few decades, due to an increase in pressure at work and in daily life, dietary changes, and *Helicobacter pylori* infections, gastric cancer has been observed to have an earlier onset[3, 4].

Gastric cancer treatment involves chemotherapy with cisplatin alone or in combination with other chemotherapeutic agents. Combination chemotherapy with cisplatin as first- or

second-line treatment for advanced and persistent gastric tumor have yielded decent responses and this treatment modality is well accepted[5]. One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor, without harming normal cells. However, chemotherapy, which induces tumor cell cytotoxicity and eventually death, has not significantly improved the overall survival of patients with gastric cancer because of poor selectivity and toxicity[6]. Therefore, it is urgent and necessary to find novel anticancer agents with potent activity and high therapeutic index to improve current treatment of gastric cancer patients.

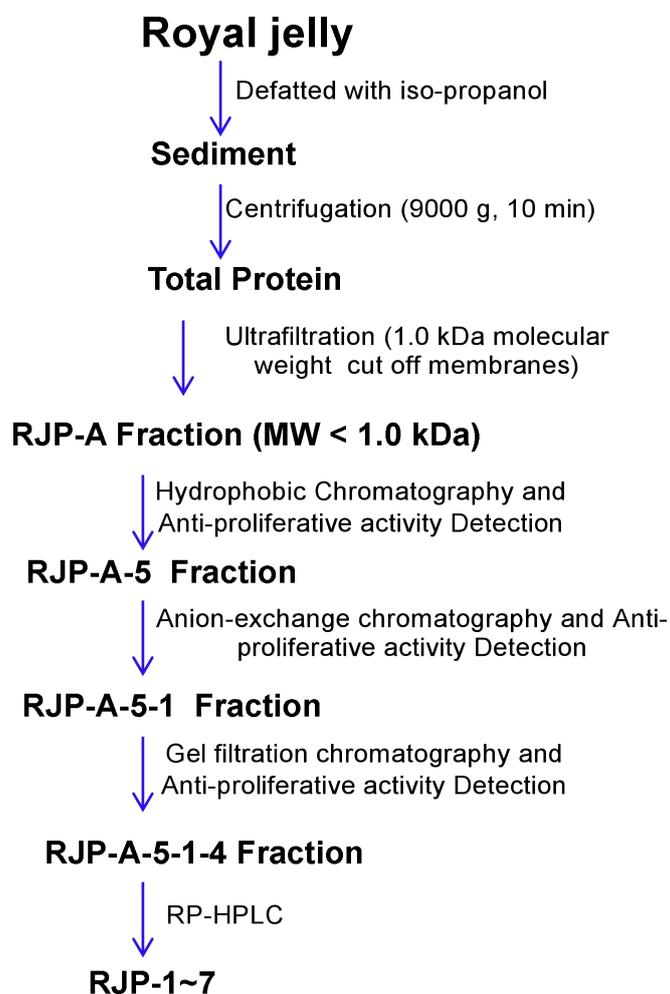
The traditional Chinese medicine (TCM) displayed an significant role in the prevention and treatment of tumors due to the advantage of proven safety. According to the previous report, most of the agents approved by the Food and Drug Administration for cancer were extracted from traditional medicine or natural sources[7]. As a traditional Chinese medicine and food, the Royal jelly is popular and is widely used in the treatments of anti-fatigue, improving the immunity, anti-aging, improving cardiovascular function, anti-cancer and anti-radiation[8]. Studies reported that the royal jelly performed significant effect in anti-fatigue[9], anti-inflammation[10], and potent anti-allergic[11] properties *in vivo*. Meanwhile, the royal jelly polysaccharide showed strong anti-oxidative activity both *in vivo* and *in vitro*. For example, the Jelleine, purified from royal jelly, showed excellent anti-microbial activities[12]. Further, the royal jelly polysaccharide also performed an interesting effect of reductions in blood pressure[13].

Although the anti-oxidant activities of royal jelly polysaccharide have been extensively studied, up to now there have been no reports concerning the biological activity of royal jelly oligopeptides (RJPs). Consequently, identifying the RJPs and exploring the anti-cancer potency will be of great value and significance. However, the potency of RJPs on protein expression in gastric cancer cells and the underlying mechanism of action are unclear. To explore the antitumor potency of RJPs on gastric cancer, we selected human gastric cancer cell lines as the research objects. Preliminary experiments have shown that the peptide isolated from the royal jelly (RJP-1~SHGEWL) can significantly inhibit the proliferation and induce apoptosis in MKN-45 cells, meanwhile, the molecular mechanisms was also explored.

2. Methods

2.1 Isolation and structural identification of RJP

The royal jelly was acquired from the Ertiantang Pharmacy (Guangzhou, China) and identified by Prof. Zhou (South China Agricultural University, Guangzhou, China). Briefly, the royal jelly and iso-propanol were mixed in a ratio of 1:10 (w/v) and stirred uninterruptedly for 5 h at 0°C. The iso-propanol was replaced every 30 min. All extraction and separation procedures were performed at 0°C. The supernatant was removed, and the sediment was freeze-dried and stored at -20°C as the total royal jelly protein. The extraction scheme of RJP is summarized in Scheme 1.



Scheme 1 Extraction scheme of RJP

2.2 Cell proliferation assay

Human gastric cancer cell lines in current study were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with RPMI 1640 medium (Gibco, Rockville) containing 10% fetal calf serum (Gibco, Invitrogen, MD, USA), 0.1 µg/L penicillin, and 0.1 µg/L streptomycin (P1400, Solarbio, Beijing, China) and were incubated in a 5% CO₂ incubator at 37°C.

The cell cytotoxicity was detected by MTT method[14]. Briefly, the cancer cells were seeded in 96-well plates (5×10^4 /well) and cultured overnight. Then, the cells were treated with different concentrations of RJPs for 24 h. After that, MTT (10 µL/well) was added and incubated for additional 4 h. The absorbance was measured (570 nm). IC₅₀ values were calculated by a nonlinear multipurpose curve fitting program (GraphPad Prism).

2.3 Cell cycle and cell apoptosis analysis

The cell cycle distribution of RJP-1-treated MKN-45 cells was determined by flow cytometry[15]. Briefly, MKN-45 cells in a logarithmic growth phase were centrifuged at 600 g for 3 min to collect cells. The cell density of the suspension was adjusted to 2.5×10^5 cells/mL, and 2 mL of the suspension was loaded into each well. After 24 h of culture, RJP-1 culture medium containing 0.07 and 0.35 mM was added, and the cultures were incubated for 24 h in the incubator. Then the cells were harvested and fixed overnight in 70% ethanol at 4°C. After fixation, the cells were centrifuged at 3000 g for 5 min to remove the ethanol. Then, the cells were washed with PBS, treated with 100 µL of RNase A, resuspended, and incubated at 37°C for 30 min in the dark. Fluorescence detection of propidium iodide (PI)-DNA complexes was determined by flow cytometry (BD FACS Calibur, Franklin Lakes, CA, USA). All the tests were repeated at least 3 times.

The Annexin V-FITC/PI double staining assay was performed according to the manufacturer's instructions[16]. Briefly, MKN-45 cells in a logarithmic growth phase were centrifuged at 600 g for 3 min to collect cells. The cell density of the suspension was adjusted to 2.5×10^5 cells/mL, and 2 mL of the suspension was loaded into each well. After 24 h of culture, RJP-1 culture medium containing 0.07 and 0.35 mM was added, and the cultures

were incubated for 24 h in the incubator. The cells were collected and centrifuged at 2000 g for 3 min at room temperature. The cells were resuspended in precooled 1× PBS and centrifuged at 2000 g for 3 min, and the cells were washed. The cells were resuspended by adding 500 µL of 1 × binding buffer. Then, 5 µL of Annexin V-FITC was added to the suspension, mixed well, and incubated for 15 min at room temperature. The cells were then stained with 5 µL of a PI staining solution before loading into a flow cytometer (BD FACS Calibur, Franklin Lakes, CA, USA). All the tests were repeated at least 3 times.

2.4 Mitochondrial membrane potential ($\Delta\psi_m$) assay

The JC-1 method was used to evaluate the mitochondrial membrane potential, and performed according to the manufacturer's instructions[17]. Briefly, MKN-45 cells in a logarithmic growth phase were centrifuged at 600 g for 3 min to collect cells. The cell density of the suspension was adjusted to 2.5×10^5 cells/mL, and 2 mL of the suspension was added to each well of a 6-well plate. After 24 h of culture, cells were treated with the RJP-1 (0, 0.07 and 0.35 mM) at 37°C for 24 h, or RJP-1 0.10 mM for 0, 4, 8, 12, 24, 48 and 60 h, respectively. CRJP was added to the cell culture medium and diluted to 10 µM, and the cells were treated for 15 min. The medium was discarded and the cells were washed once with PBS. Then 1 mL of the cell culture media and 1 mL of JC-1 staining working solution was added and mixed well, and subsequently incubated at 37°C for 20 min. After incubation, the cells were collected and centrifuged at 2000 g for 3 min at room temperature. The cells were resuspended in prechilled 1 × JC-1 staining buffer, centrifuged at 2000 g for 3 min, and washed twice. The cells were resuspended by the addition of 500 µL of JC-1 staining buffer and then loaded onto a flow cytometer (BD FACS Calibur, Franklin Lakes, CA, USA).

2.5 Western blotting analysis

The MKN-45 cells were plated in 6-well culture dishes, and treated with RJP-1 (0.07 and 0.35 mM) for 24 h. Total cellular protein lysate as well as subcellular fractions, cytosolic and mitochondrial fractions, were prepared as described previously. Nuclear extract was prepared using the nuclear extract kit according to manufacturer's protocol. Protein concentration was determined using BCA protein assay kit. Cell lysates (50 µg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with antibodies against target

proteins[18]. The bands were visualized and β -actin protein was used as the internal control.

2.6 Statistical Analysis

Experiments were repeated at least three times and results are expressed as mean \pm SD. Data were analyzed by Student's *t*-test and an analysis of variance (ANOVA) test followed by a Tukey post test to determine the significant differences between groups. $p < 0.05$ were considered to be significant. All statistical analyses were performed with GraphPad-Prism 5 (San Diego, USA).

3. Results and Discussion

3.1 *In Vitro* antigastric cancer potency of RJPs

Currently, chemotherapy is a mainly approach for the treatment of gastric cancer, and drug resistance is one of the most significant obstacles in chemotherapy. Besides, toxicities of using high doses of chemotherapeutic agents to get over drug resistance also significantly impede patients' recovery[19]. Therefore, it is urgent and necessary to find novel anticancer agents with potent activity and high therapeutic index to improve current treatment of gastric cancer patients. The traditional Chinese medicine (TCM) displayed an significant role in the prevention and treatment of tumors due to the advantage of proven safety[20]. Finally, seven novel RJPs were isolated and their amino acid sequences were identified.

The peptide is usually protonated under ESI-MS/MS conditions, and fragmentations mostly occur at the amide bonds because it is difficult to break the chemical bonds of the side chains at such low energy. Therefore, the b and y ions are the main fragment ions when the collision energy is < 200 eV[21]. The RJP-1 was analyzed by HPLC-ESI-MS for molecular mass determination and peptide characterization (**Figure 1**).

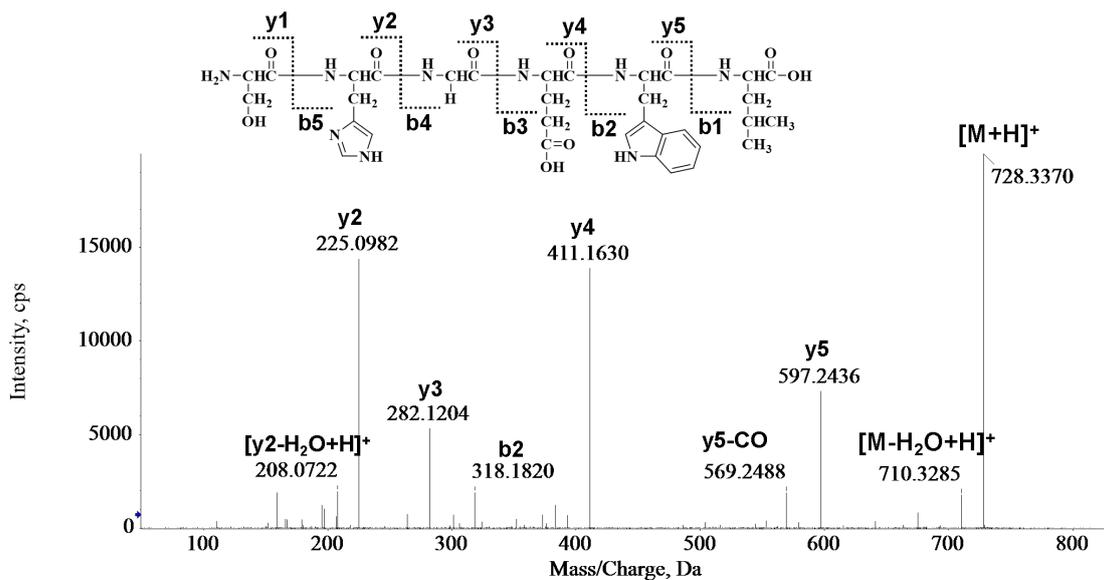


Figure 1 MS spectrum and structure of RJP-1

HPLC-ESI-MS was carried out on a SCIEX X500R Q-TOF mass spectrometer (Framingham, U.S.A.). And the MS conditions were as follows: ESI-MS analysis was performed using a SCIEX X500R Q-TOF mass spectrometer equipped with an ESI source. The mass range was set at m/z 100-1200. The Q-TOF MS data were acquired in positive mode and conditions of MS analysis were as follows: CAD gas flow-rate, 7 L/min; drying gas temperature, 550°C; Ion spray voltage, 5500 V; Declustering potential, 80 V. Software generated data file: SCIEX OS 1.0. Further, the purity ($> 97.6\%$) was determined by HPLC. The RJPs were dissolved in PBS as a stock solution and stored at -20°C.

The ion fragment m/z 728.3370 was regarded as $[M+H]^+$. The ion m/z 710.3285 was regarded as the $[M-H_2O+H]^+$ fragment, while m/z 597.2436 was regarded as the y_5 ion, m/z 569.2488 was regarded as the $[y_5-CO]^+$ fragment, m/z 411.1630 was regarded as the y_4 ion and m/z 318.1820 was regarded as the b_2 ion. The ion (m/z 282.1204) was the y_3 ion, and ion (m/z 225.0982) was the y_2 ion, m/z 208.0722 was the $[y_2-H_2O+H]^+$ fragment, m/z 110.0705 was the $[His-COOH+H]^+$ fragment. On the basis of this, we concluded that the sequence of the peptide was SHGEWL. The rest of the RJPs were identified as RJP-1, and the amino acid sequences are list in **Table 1**. Further, the purity was detected by HPLC $>98\%$. The RJPs were diRJP1ved in DMSO (4.0 mM) and stored at 4°C as a stock solution.

Table 1 Inhibition of proliferation in gastric cancer cells of RJPs

RJPs	Amino Acid Sequence	IC ₅₀ (mM)		
		MKN-45	SGC-7901	MGC-803
RJP-1	SHGEWL	0.07 ± 0.01	0.25 ± 0.03	0.29 ± 0.03
RJP-2	EHGEYE	1.56 ± 0.13	1.74 ± 0.14	> 2.0
RJP-3	FRHALS	1.12 ± 0.10	1.05 ± 0.11	0.88 ± 0.06
RJP-4	EGHGF	1.04 ± 0.12	1.32 ± 0.13	1.46 ± 0.13
RJP-5	WEHKHA	> 2.0	1.76 ± 0.14	> 2.0
RJP-6	LWEHSH	1.13 ± 0.14	1.66 ± 0.11	1.44 ± 0.12
RJP-7	KYGHEHS	1.84 ± 0.14	1.63 ± 0.15	> 2.0

IC₅₀ values are shown as mean ± standard error of the mean (SD), from at least three independent experiments. The three cancer cell lines were seeded into 96-well plates, and cells were treated with different concentrations of RJPs for 24. The survival rate of cells treated with RJPs was measured by the MTT method.

To study the effect of RJPs on gastric cancer *in vitro*, we treated the three gastric cancer cell lines (SGC-7901, MKN-45 and MGC-803) with different concentrations of RJPs for 24 h. The activity of the cancer cells after RJPs treatment was detected by the MTT assay. As shown in **Table 1**, most of the RJPs performed anti-proliferative activity against the three human gastric cancer cell lines. Interestingly, RJP-1 potently inhibited the growth of SGC-7901, MKN-45 and MGC-803 cells, with IC₅₀ values of 0.07±0.01, 0.25±0.03, 0.29±0.03 mM, respectively. The inhibition rate was significantly higher in MKN-45 cells after 24 h of treatment with RJP-1. Since RJP-1 was indicated enhancing the anti-proliferative activity in MKN-45 cancer cells, together with the fact that gastric cancer is one of the most common malignancies. This is the reason why we chose MKN-45 cells to explore the anti-cancer potency and the underlying mechanism.

Further, the cytotoxicity of the RJP-1 in normal cells from healthy tissue was also evaluated.

The cytotoxicity of RJP-1 in human gastric epithelial cell line GES-1 is at much higher level compared with the tumor cells, in which the IC_{50} value is 1.93 ± 0.15 mM to GES-1 cells.

3.2. RJP-1 induces cell cycle arrest at the G0/G1 phase by regulating the cyclins expression

Checkpoints are important regulatory nodes of the cell cycle. Cells can only enter the next cell cycle after passing these checkpoints[22]. The function of the G0/G1 phase detection point is to integrate and transmit complex intracellular and extracellular signals, such as various growth factors, mitogens, and DNA damage, as well as to determine whether cells are undergoing division and apoptosis. Deregulation of cell cycle progression is a common feature of cancer cells. Therefore, targeting the regulatory cyclins has been proposed as an important strategy for the treatment of human malignancies[23]. When the cells begin to synthesize DNA during G1/S conversion, CDK2 will combine with its regulatory subunit cyclin E to form a CDK2/Cyclin E complex, leading to Rb phosphorylation, and then E2F factor is released and cells are accelerated into the S phase[24].

To further investigate the inhibitory effect of RJP-1 on the proliferation of MKN-45 cells, we evaluated the cell cycle distribution of MKN-45 cells after 24 h of treatment with RJP-1. The percentage of cells in G0/G1 phase increased from 38.0% to 53.7% and 60.7%, respectively(**Figure 2A**). Flow cytometry analysis showed that the proportion of MKN-45 cells that were at the G0/G1 phase significantly increased after treatment with RJP-1 compared to the control group.

Further, we evaluated the expressions of CDK2, CDK6, cyclin D and cyclinE after the RJP-1 treatments. Of interest, RJP-1 induced cell cycle arrest by down-regulating the expressions of cyclin D, cyclin E, CDK2 and CDK6, suggesting that these proteins are involved in cell cycle progression in MKN-45 cells (**Figure 2B**). These results indicated that RJP-1 could effectively induce cell cycle arrest at the G0/G1 phase by inhibiting the cyclins expression.

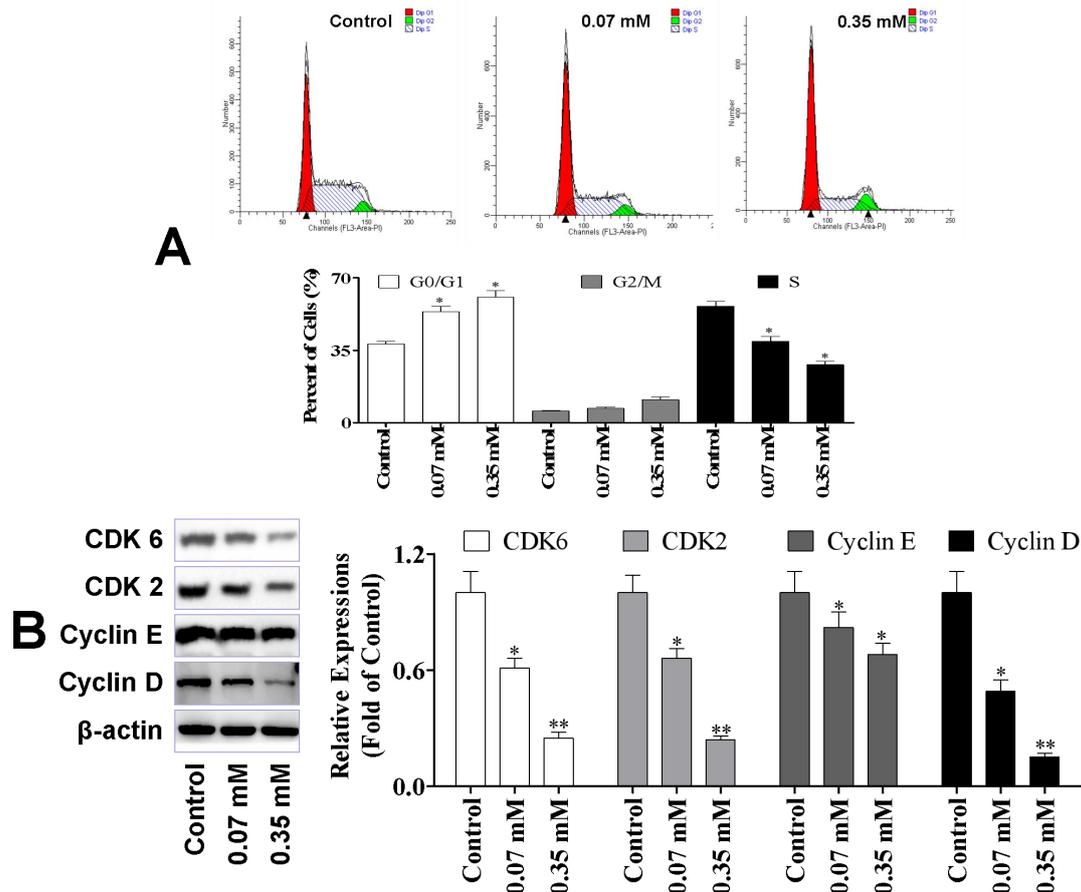


Figure 2 RJP-1 induced G0/G1 cell cycle arrest in MKN-45 cells

(A) Flow cytometry was used to evaluate the cell cycle distribution of MKN-45 cells under the RJP-1 treatments; (B) Western blotting was performed to detect cyclin D, cyclin E, CDK2 and CDK6 expressions. β -actin is used as a loading control. Statistical analysis of the expressions of cyclins in RJP-1 treated MKN-45 cells. The ratio of protein level was normalized according to the value of the control. All data are expressed as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control.

3.3 RJP-1 regulated the p53/MDM2 state and the ATM/p21 signaling pathway

The cell cycle refers to the entire process of continuous cell division. When cell cycle arrest occurs during cell division, it is often due to damage or errors that are difficult to repair during cell division. This theory suggests that RJP-1-induced G0/G1 arrest may be due to DNA damage that is difficult to repair in cells. The ATM/ATR signaling pathway can repair damaged DNA by modulating the activity of various proteins. At present, it is generally believed that the ATM/ATR signaling pathway mediates G0/G1 arrest by regulating the

expression of p53[25]. ATM can directly regulate p53, which increases the p53 protein level, which in turn enhances p21 transcription. Activation of ATM during DNA damage can upregulate the expression of the p21 protein and downregulate p53 protein expression. Finally, the formation of the CDK2/cyclin E complexes is inhibited, and the cell cycle is arrested at the G0/G1 phase[26]. Further, the p53 is a tumor suppressor which plays a pivotal role of inducing cell cycle arresting, DNA repair, senescence, and apoptosis. While MDM2 (murine double minute 2) is the main endogenous negative regulator of p53. This oncoprotein MDM2 binds p53 and negatively regulates p53 activity by direct inhibition of p53 transcriptional activity and enhancement of p53 degradation via the ubiquitin-proteasome pathway[27]. An appealing therapeutic strategy for many wild-type p53 tumors with over expressed MDM2 is to restore p53 activity via inhibiting the p53/MDM2 interaction. The p21 is potent cyclin-dependent kinase inhibitors that bind to and inhibit the activities of CDKs; thus, increased levels of these proteins thus indicate the induction of G0/G1 cell cycle arrest. Therefore, we detected whether the RJP-1 regulate the p53 and MDM2 expressions. Further, the protein expression levels of ATM and p21 in MKN-45 cells under RJP-1 treatments were also evaluated.

To our expect, the p21 and ATM expressions were up-regulated in RJP-1-treated MKN-45 cells, indicating that RJP-1 is related to p21-dependent cell cycle arrest (**Figure 3**). Further, RJP-1 up-regulated p53 expression; while in the other hand, it down-regulated MDM2 expression, accordingly (**Figure 3**). Treatment of 0.35 mM RJP-1 changed the p53/MDM2 ratio from 0.21 (Control group) to 2.19. Based on the results, we inferred that RJP-1 may also regulate the p53/MDM2 state to promote the MKN-45 cell apoptosis.

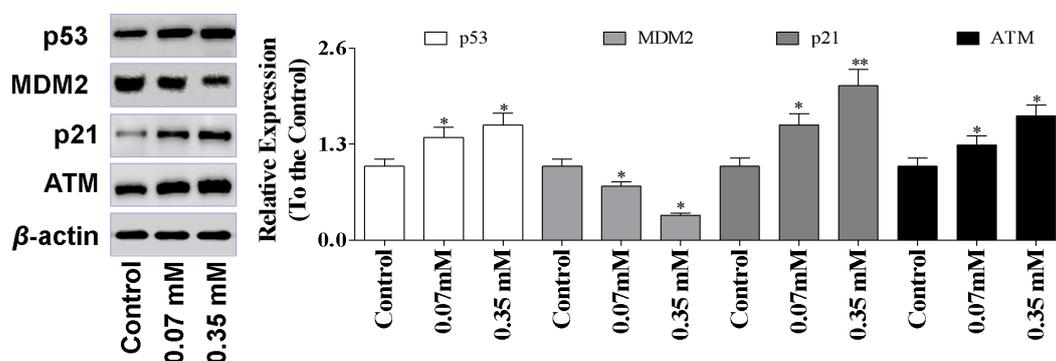


Figure 3 RJP-1 regulated the p53/MDM2 state, and ATM, p21 protein expressions

Western blotting was used to detect the protein expression levels in MKN-45 cells after 24 h of treatment with RJP-1. The ratio of protein levels was normalized according to the values of the control. All data are expressed as the mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01 compared to the control.

3.4 RJP-1 promotes caspase-dependent apoptosis in MKN-45 cells

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. The process of apoptosis plays key role in the development of most of the cancers. It has been reported that tumors subjected to radiation and cytotoxic agents showed increased rates of apoptosis, implying that enhanced rate of apoptosis can be used in cancer therapy[28].

To explore whether the inhibition of the proliferation potency of RJP-1 is related to cell apoptosis, we analyzed the apoptotic rate of RJP-1-treated MKN-45 cells using an Annexin V FITC/PI double staining kit and flow cytometry. We found that the percentage of apoptotic cells increased significantly with RJP-1 treatments, from 3.42% to 21.1% and 34.1%, respectively (**Figure 4A**). These results indicated that RJP-1 significantly induced MKN-45 cells apoptosis.

Caspase activation plays a central role in the execution of apoptosis, we then evaluated whether the Caspase activation is involved in RJP-1 induced apoptosis. As shown in **Figure 4B**, the expressions of Cleaved caspase-9 and Cleaved caspase-3 were significantly up-regulated; at the same time, the expressions of Caspase-9 and Caspase-3 were significantly down-regulated in RJP-1-treated cells. Further, the Cleaved-PARP was also strongly activated in RJP-1-treated MKN-45 cells.

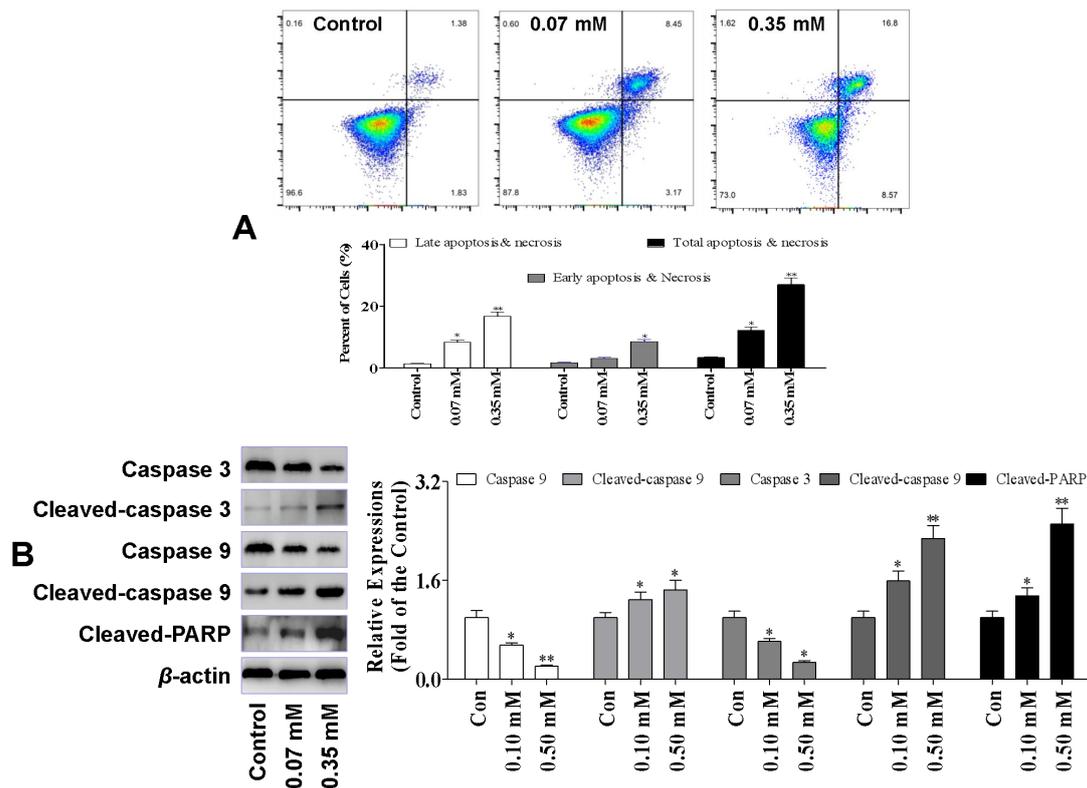


Figure 4 RJP-1 induced cell apoptosis accompanied with the caspases activation

(A) After 24 h of treatment with RJP-1, the Cells were stained with Annexin-V and PI, and detected by flow cytometry. Representative scatter diagrams. In each scatter diagram, the abscissa represents the fluorescence intensity of the cells dyed by Annexin V; and the ordinate represents the fluorescence intensity of the cells dyed by PI. The lower left quadrant shows the viable cells, the upper left shows necrotic cells, the lower right shows the early apoptotic cells; while the upper right shows late apoptotic cells. (B) RJP-1 activates Caspase cleavage in a concentration-dependent manner. After the MKN-45 cells were treated as the experiment design, the Cleaved-PARP, Caspase 3, Cleaved-caspase 3, Caspase 9 and Cleaved-caspase 9 expression levels were detected by Western blotting. β -actin was used as an internal control. The data are expressed as the mean \pm SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, compared with the control group.

3.5 RJP-1 induced apoptosis through mitochondrial pathways

The mitochondrion-dependent pathway is one of the most common apoptotic pathways in vertebrate tumor cells. Mitochondrial membrane permeability increases and mitochondrial transmembrane potential ($\Delta\Psi_m$) decreases when the ratio of Bcl-2/Bax decreases[29].

Mitochondrial dysfunction, as indicated by the dissipation of $\Delta\Psi_m$, could subsequently cause the release of cytochrome c (Cyt c) from mitochondria into the cytosol. Cytochrome C in the cytoplasm activates Apaf-1 and caspase-9 and -3, which cleaves DNA and produces apoptotic bodies that ultimately lead to cell apoptosis[30]. In the above experiments, we observed that RJP-1 could effectively induce apoptosis in MKN-45 cells accompanied with the Caspases activation (**Figure 4B**). To explore the intrinsic mechanism of this effect, we performed western blotting analysis to identify the core proteins associated with apoptosis in the mitochondrial apoptotic signaling pathway.

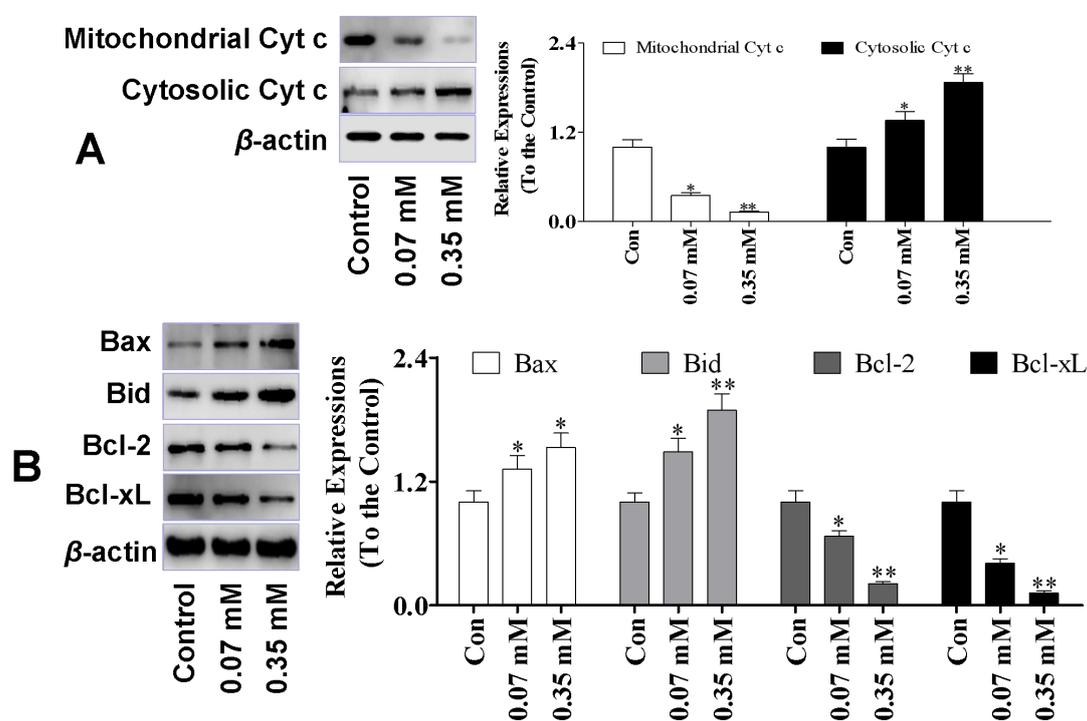


Figure 5 Effect of RJP-1 on the expression of signaling molecules in the mitochondrial apoptotic pathway

(A) After 24 h of treatment with RJP-1, MKN-45 cells were collected. Western blotting was performed to detect cytosolic and mitochondrial levels of the pro-apoptotic proteins cytochrome c. (B) MKN-45 cells were treated with various concentrations of RJP-1. Western blotting was performed to detect the levels of Bcl-2, Bcl-xL, Bid and Bax. The ratio of protein levels was normalized according to the values of the control. All data are expressed as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control.

The results showed that RJP-1 treatments resulted in a striking up-regulation of cytosolic Cyt

c and down-regulation of mitochondrial Cyt c compared with the control group (**Figure 5A**). Further, we investigated the expression levels of the Bcl-2 family of apoptosis regulator proteins. We found that RJP-1 significantly increased the protein expression levels of Bad and Bax, and decreased that of Bcl-2 and Bcl-xL relative to the control group (**Figure 5B**).

3.6 RJP-1 on the Mitochondrial Membrane Potential ($\Delta\psi_m$) of MKN-45 Cells

The balance of $\Delta\psi_m$ and mitochondrial integrity is significant for the physiological function of cells. The collapse of $\Delta\psi_m$ is correlated to the events of apoptotic process. When the concentration of ROS and oxidative stress reach a certain level, the $\Delta\psi_m$ would be changed, resulting in the release of apoptosis factors[31]. In current study, the JC-1 fluorescent probe was applied to detect the potency of RJP-1 on the $\Delta\psi_m$. As shown in **Figure 6A**, the RJP-1 treatment induced a clearly $\Delta\psi_m$ decrease signal, and played in a dose- and time-dependent manner.

The thioredoxin (Trx) system plays a pivotal role in the regulation of cellular reduction-oxidation (redox) homeostasis, further, over-expression of Trx and TrxR were founded in most of the cancer cells[32]. As shown in **Figure 6B**, the both the Trx and TrxR expressions were down-regulated by RJP-1, suggesting that this effect may be part of the mechanism of action for RJP-1.

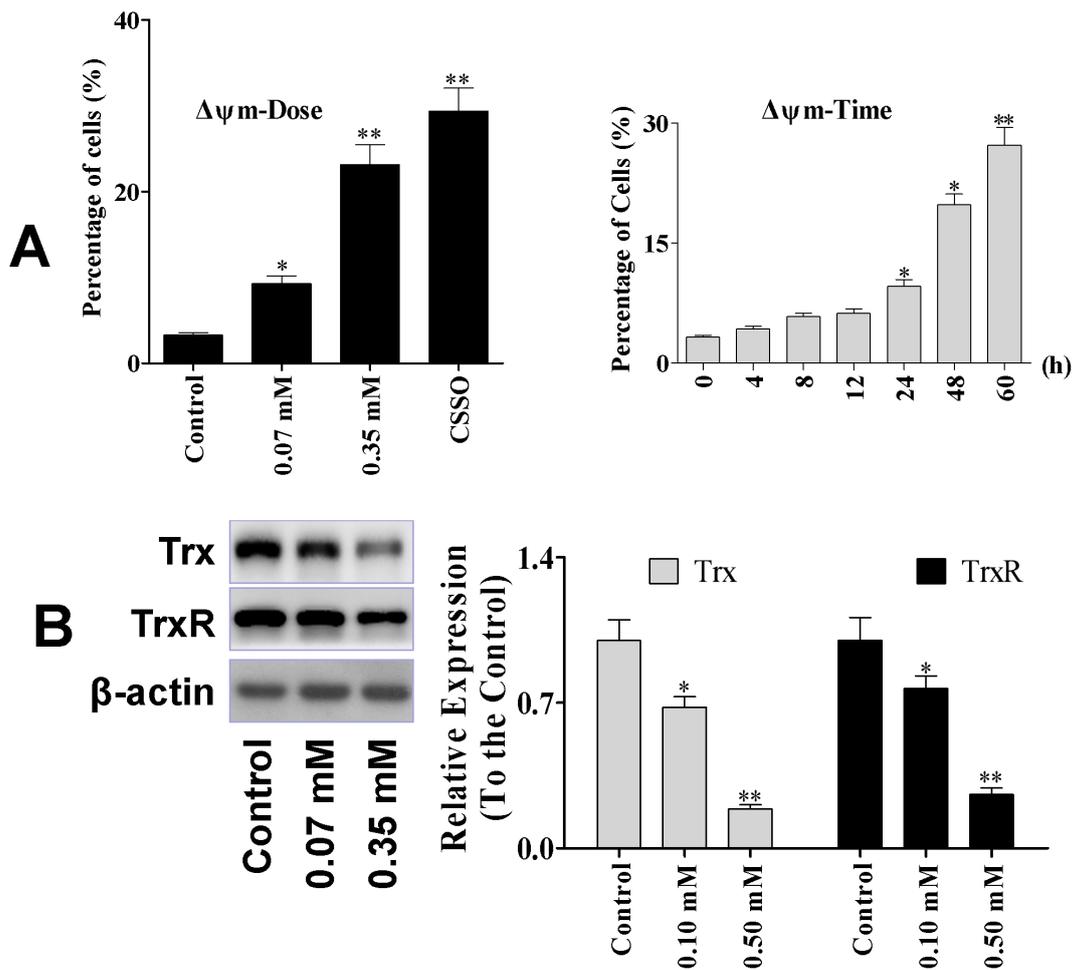


Figure 6 RJP-1 induced $\Delta\psi_m$ collapse and suppressed the Trx system

(A) The decline of $\Delta\psi_m$ in MKN-45 cells with RJP-1 treatment was detected by JC-1 staining. Briefly, the MKN-45 cells were treated as the experimental design. After that, the $\Delta\psi_m$ was detected by JC-1 staining according to the manufacturer's protocol (Beyotime, China) and assayed by flow cytometry (BD FACS Calibur, Franklin Lakes, CA, USA). (B) After 24 h of treatment with RJP-1, MKN-45 cells were collected. Western blotting was performed to detect the levels of Trx and TrxR. The ratio of protein levels was normalized according to the values of the control. All data are expressed as the mean \pm SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to the control.

4. Conclusion

Of note, we demonstrated that the Royal Jelly peptide-1 (RJP-1-SHGEWL) performed significant growth-inhibitory potency on gastric cancer cell lines *in vitro*. We revealed that RJP-1 induced MKN-45 cell apoptosis through the Caspase-dependent mitochondrial pathway, and via G0/G1 phase block by regulating G0/G1 checkpoint proteins. We also found that RJP-1 significantly regulated the state of p53/MDM2, and activated the mitochondrion-dependent pathway. However, further studies are required to identify the specific molecular targets and the signaling pathways by RJP-1 treatment. In addition, whether RJP-1 exhibits the similar effects on other cellular systems still remains to be further investigated. Nevertheless, our results shed some light on the mechanisms of RJP-1 inducing gastric cancer cells apoptosis.

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Conflict of interest

The authors declare that they have no conflicts of interest concerning.

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