

Paeonol exerts anti-tumor activity against colorectal cancer cells by inducing G₀/G₁ phase arrest and cell apoptosis via inhibiting the Wnt/ β -catenin signaling pathway

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Received November 18, 2019; Accepted May 19, 2020

DOI: 10.3892/ijmm.2020.4629

Abstract. Paeonol is a simple phenolic compound isolated from herbal root bark, which has been reported to possess numerous biological and pharmacological characteristics, including a desirable anti-tumor effect. To date, the effect of paeonol against colorectal cancer (CRC) cells is yet to be fully elucidated. Therefore, the present study aimed to identify the underlying mechanism via which paeonol exerts its anti-tumor activity on HCT116 cells. After incubation with various concentrations of paeonol (7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 μ g/ml), the inhibitory effect of paeonol on cell viability was assessed using a Cell Counting Kit-8 assay. Cell apoptosis and cell cycle distribution were measured using flow cytometry. Moreover, caspase activity was measured using a colorimetric caspase assay. Luciferase assay was also used to determine the β -catenin-mediated transcriptional activity of T-cell specific transcription factor/lymphoid-enhancer binding factor (TCF/LEF), and western blotting analysis was performed to measure the related expression of proteins. The results indicated that paeonol exhibited a notable effect against HCT116 cells by inducing G₀/G₁-phase arrest, as demonstrated by downregulation of the cell cycle regulators cyclin-dependent kinase 4 and cyclin D1 and upregulation of p21^{Cip1} in a dose-dependent manner. Furthermore, paeonol dose-dependently induced cell apoptosis, accompanied by an increase in the Bax/Bcl-2 ratio, release of cytochrome c and further activation of caspases. Paeonol also dose-dependently blocked the activation of the Wnt/ β -catenin signaling pathway by suppressing the expression of β -catenin, resulting in a

decrease in β -catenin-mediated activity of TCF/LEF and downregulation of downstream target genes, including cyclin D1, survivin and c-Myc. Therefore, the present results suggested that paeonol exerted its anti-tumor effects on CRC cells, including the inhibition of cell proliferation, induction of cell cycle arrest and initiation of apoptosis, at least partly by suppressing the Wnt/ β -catenin pathway, which may offer a promising therapeutic strategy for CRC.

Introduction

Colorectal cancer (CRC), a type of malignant gastrointestinal tumor, is the third leading cause of tumor-associated mortality worldwide (1). Moreover, in China, the CRC incidence in 2018 was 12.8% for men and 11.3% for women (2), and this rate is rapidly increases along with the development of the Chinese economy (3). Currently, the primary curative treatment for CRC is surgical resection; however, adjuvant chemotherapy has been incorporated to reduce high rates of adjacent tissue invasion and metastasis, thus decreasing the relapse rate (4). Due to the invariable incidence of drug resistance and serious side effects, including diarrhea, nausea, swelling, vomiting, abdominal pain, tiredness, low blood levels of albumin and other abnormalities, associated with standard anti-cancer drugs, the outcomes of chemotherapy and other effective measures are currently unsatisfactory for patients with CRC (5). Therefore, the investigation of novel treatment strategies with a safe profile that act via different signaling pathways is urgently required to develop improved targeted therapies.

Contrarily to traditional chemotherapeutic drugs, certain natural products, including flavonoids and jatrorrhizine (4), are considered to be potential candidates for neoplastic therapy on account of their substantial biological activities and relatively low adverse effects (6). Moreover, ongoing research for anti-cancer agents from medicinal plants has led to the examination of Traditional Chinese Medicine (7). As a simple phenolic compound extracted from the herbal root bark, paeonol (2'-hydroxy-4'-methoxyacetophenone) has substantial biological and pharmacological properties, including significant sedation, analgesic action, antipyresis, anti-inflammation, anti-oxidation, anti-hypertension, neuroprotection and

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Key words: paeonol, colorectal cancer, apoptosis, cell cycle, Wnt/ β -catenin signaling pathway

immunomodulation (8,9). In addition, paeonol has attracted increased attention in recent years due to its desirable anti-tumor effect against various types of cancer cell, both *in vitro* and *in vivo*, as revealed by its ability to inhibit cell proliferation and induce cell apoptosis (8,10).

Previous studies have reported that several signaling pathways have important roles in the progression of cancer, including NF- κ B, C-X-C motif chemokine ligand 4/C-X-C motif chemokine receptor 3B and the PI3K/Akt/NF- κ B pathway (10-12). It has been shown that the role of the canonical Wnt signaling pathway is to regulate its downstream genes responsible for the cell cycle and cell survival (13). In addition, it is crucial to maintain homeostasis in multiple tissues throughout the body via the Wnt/ β -catenin pathway, and dysregulation of this pathway is regarded as an important oncogenic event in numerous types of human tumor, particularly in the initiation and progression of CRC (14). As aberrant activation of the Wnt/ β -catenin pathway is involved in cell proliferation, invasive features and development of chemoresistance resistance in cancer cells, this pathway is considered as a potential novel target of chemotherapeutic or chemopreventive agents for cancer types (15). Currently, several natural agents from drug discovery platforms, including aesculetin (16), baicalin (17), hydrnocarpin (15), isobavachalcone (18), jatrorrhizine (4), *Sanguisorba officinalis* (19) and wogonin (20), have been verified to directly or indirectly target β -catenin and its downstream signaling partners T cell-specific transcription factor/lymphoid-enhancer binding factor (TCF/LEF), consequently reducing the viability of CRC cells. However, the underlying mechanisms responsible for the effects of paeonol against CRC are yet to be fully elucidated. Therefore, the present study aimed to identify the mechanisms of the anti-tumor effect of paeonol on human CRC cells.

Materials and methods

Major reagents. Paeonol (purity, >98%) was obtained from Sigma-Aldrich (Merck KGaA; cat. no. H35803) and the stock solution of paeonol in alcohol was diluted to obtain the required concentrations (7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 μ g/ml). RPMI-1640 medium and FBS were provided by Thermo Fisher Scientific, Inc. A Cell Counting Kit-8 (CCK-8) was obtained from Beyotime Institute of Biotechnology. The TRIzol[®] total extraction kit was from Invitrogen (Thermo Fisher Scientific, Inc.). Ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma-Aldrich (Merck KGaA). The Annexin-V-FITC/PI apoptosis detection kit was from BD Biosciences. Colorimetric caspase assay kits were obtained from Abcam [cat. nos. ab39401 (caspase-3), ab39700 (caspase-8) and ab65608 (caspase-9)]. The TCF/LEF reporter plasmid (cat. no. GM-021042) was purchased from Jiman Biotechnology (Shanghai) Co., Ltd. Micropoly-transfecter (cat. no. MT103) was obtained from Biosky Biotechnology Corporation and D-Luciferin sodium (cat. no. 7902-100) was from BioVision, Inc. RIPA buffer (cat. no. 6505729) and the bicinchoninic acid (BCA) Protein Assay kit (cat. no. BL52A-1) were obtained from Biosharp Life Sciences.

The primary rabbit antibodies against human Bax (cat. no. ab32503), Bcl-2 (cat. no. ab59348), p21^{Cip1} (cat. no. ab145), cytochrome C (cat. no. ab13575), cyclin D1 (cat. no. ab226823), cyclin-dependent kinase (CDK)4 (cat. no. ab137675), c-Myc

(cat. no. ab12213), survivin (cat. no. ab76424), glycogen synthase kinase (GSK)-3 β (cat. no. ab32391), β -catenin (cat. no. ab32572) and β -actin (cat. no. ab8229) were obtained from Abcam. In addition, horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG antibodies (cat. no. SA00001-1 or SA00001-2) were obtained from ProteinTech Group, Inc. The other chemicals were of analytical grade and obtained from local reagent suppliers.

Cell line and culture. The human CRC HCT116 cell line was provided by the Cell Bank of the Chinese Academy of Sciences and was cultured in RPMI-1640 medium containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 95% air and 5% CO₂. The cells used in the experiments were in the logarithmic growth phase.

Cell proliferation assay. The CCK-8 assay was performed to determine the number of viable cells according to the manufacturer's protocol. In brief, 5x10³ HCT116 cells per well in a 96-well plate were incubated at 37°C with a series of concentrations of paeonol (0, 7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 μ g/ml) for 12, 24, 48 and 72 h. Each condition was set up in 6-wells and the assay was performed in duplicate. Then, 10 μ l CCK-8 solution was added to each well of the plates at 12, 24, 48 and 72 h. After incubation at 37°C for another 4 h, the absorbance (A) at 550 nm was detected to determine the number of viable cells using a microplate reader (iMark680; Bio-Rad Laboratories, Inc.). The inhibitory rate (IR) of HCT116 cells was calculated as follows: IR (%) = [(mean A_{control} - mean A_{blank}) - (mean A_{test} - mean A_{blank})] / (mean A_{control} - mean A_{blank}) x 100%, and the IC₅₀ was obtained from the cell growth curve using Bliss software (version 2.0; Bliss Software Technologies Inc.).

Analysis of cell cycle. Based on the IC₅₀ value, different doses of paeonol (20, 40 and 80 μ g/ml) were selected for the study. After incubation at 37°C with paeonol in a 6-well plate (1x10⁵ cells per well) for 12, 24 and 48 h, the cells were harvested, washed with 1X PBS and then incubated with 50 μ g/ml PI solution containing 0.1 mg/ml RNase A in PBS (pH 7.4) for 30 min at room temperature in the dark. Subsequently, flow cytometry (FCM) was performed using a FACSCalibur (BD Biosciences) to analyze the fluorescence of the PI-DNA complex and further to quantify cell-cycle fractions from $\geq 1 \times 10^4$ cells using Cell Quest software (version 3.3; BD Biosciences).

Determination of apoptosis. The percentage of early apoptosis (Annexin V⁺/PI⁻) and late apoptosis (Annexin V⁺/PI⁺) was detected by FCM according to our previous study (7). 20, 40 and 80 μ g/ml paeonol at an earlier time point (24 h) and a moderate dose of paeonol (40 μ g/ml) at 12, 24 and 48 h were selected. After incubation at 37°C, the cells were collected and washed three times with ice-cold PBS. Following staining with 5 μ l Annexin V-FITC and 5 μ l PI in 100 μ l 1X binding buffer for 15 min at room temperature in the dark, the total apoptotic rate was examined with a flow cytometer using Cell Quest software (version 3.3; BD Biosciences).

Colorimetric caspase activity assay. Following the manufacturer's instructions, colorimetric caspase assay kits were performed to measure the caspase activity. In brief, 1x10⁶

HCT116 cells/ml were incubated at 37°C with 0, 20, 40 and 80 µg/ml paeonol for a moderate period of time (48 h). After washing with ice-cold PBS, ice-cold cell lysis buffer was used to lyse cells for 15 min and then the supernatant was separated by centrifugation (12,000 x g at 4°C for 10 min). The cell lysate was added to assay plates containing reaction buffer with 10 µl acetyl-Asp-Glu-Val-Asp p-nitroanilide as a substrate for caspase-3, acetyl-Ile-Glu-Thr-Asp p-nitroanilide for caspase-8 or acetyl-Leu-Glu-His-Asp p-nitroanilide for caspase-9, followed by incubation at 37°C in the dark for 1.5 h. Finally, the A at 405 nm was measured with a microplate reader to quantify the formation of p-nitroanilide, and the relative increases of caspase-3, -8 and -9 activity were calculated by comparing the A of paeonol-treated HCT116 cells with the control group.

TCF/LEF luciferase reporter assay. The TCF/LEF dual-luciferase reporter assay was performed following the manufacturer's instructions with minor modifications. In brief, 1x10⁴ HCT116 cells/well were seeded into 24-well microtiter plates and maintained in RPMI-1640 medium overnight at 37°C in an incubator with 5% CO₂ prior to transfection. After incubation at room temperature for 10 min, the mixture of 2 µg TCF/LEF reporter plasmid and 2 µl micropoly-transfecter was added to RPMI 1640 culture medium with HCT116 cells. Following the antibiotic screening for 24 h, the transfection efficiency of HCT116 cells was performed by measuring the signals from TCF/LEF reporter (firefly luminescence). Then, the transfected cells were incubated at 37°C with either 0 (control), 20 or 80 µg/ml paeonol for 48 h. Finally, D-luciferin sodium at a final concentration of 15 mg/ml was added to each well to quantify the luciferase activity and the fluorescence images were acquired using the IVIS[®] Spectrum system and Living Image[®] software (version 4.5; IVIS[®] Spectrum; PerkinElmer, Inc.).

Western blot analysis. Following incubation with 20, 40 and 80 µg/ml paeonol at 37°C for 48 h, RIPA buffer was used to extract proteins from the harvested cells and then a BCA Protein Assay kit was used to determine the protein concentration in the supernatant after centrifugation at 12,000 x g and 4°C for 30 min. Aliquots containing 10 µg protein per lane were subjected to 10% SDS-PAGE and the separated proteins were transferred onto PVDF membranes (EMD Millipore). After blocking with a mixture of 5% skimmed milk/0.1% Tris-buffered saline containing 0.1% Tween-20 (TBST) at 25°C for ≥2 h, the membranes were probed with anti-CDK4, anti-p21^{Cip1}, anti-Bax, anti-Bcl-2, anti-cytochrome C, anti-glycogen synthase kinase 3 β (GSK-3β), anti-c-Myc (all 1:1,000), anti-cyclin D1 (1:2,000), anti-survivin, anti-β-catenin (both 1:5,000) and mouse anti-β-actin (1:1,000) on a shaker table at 4°C for ≥12 h, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:2,000) at room temperature for 2 h. After further rinsing with TBST three times, the membranes were visualized using an enhanced chemiluminescence substrate (Amersham; Cytiva). The intensity of each band relative to β-actin was determined semi-quantitatively using ImageQuant TL software (version 7.0; Cytiva) (21).

Statistical analysis. Data are presented as the mean ± standard deviation of ≥3 independent experiments performed in

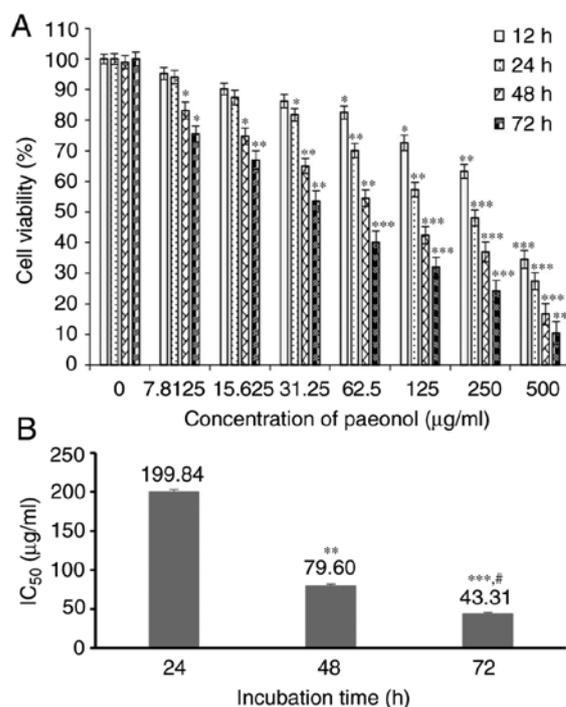


Figure 1. Effect of paeonol on the viability in HCT116 cells. (A) After incubation with different doses of paeonol for 12, 24, 48 and 72 h, the cell viability was assessed with a Cell Counting Kit-8 assay. (B) Histogram of IC₅₀ for various durations. *P<0.05, **P<0.01 and ***P<0.001 vs. control group (0 µg/ml); #P<0.05 vs. the group at the time point of 48 h.

triplicate. Comparisons between two groups were analyzed with an unpaired Student's t-test, and one-way ANOVA followed by Tukey's post hoc test was performed to determine differences among >2 groups using SPSS 18.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Paeonol reduces the number of viable HCT116 cells. After incubation with paeonol for various intervals (12-72 h), it was demonstrated that paeonol significantly suppressed the proliferation of HCT116 cells, and the number of viable cells was inhibited with increasing concentrations of paeonol and the prolongation of incubation time (P<0.05; Fig. 1A). Moreover, the IC₅₀ of paeonol was determined as 199.84 µg/ml at 24 h, 79.60 µg/ml at 48 h and 43.31 µg/ml at 72 h (Fig. 1B). Collectively, these results suggested that paeonol reduced the number of viable HCT116 cells in a dose- and time-dependent.

Paeonol induces G₀/G₁-phase arrest in HCT116 cells. As cell proliferation is closely regulated by the cell cycle (22), FCM was used to assess whether the effect of paeonol on inhibiting cell proliferation was due to induction of cell cycle arrest. Following incubation with 0, 20, 40 and 80 µg/ml paeonol for 48 h, the FCM results indicated that the proportion of HCT116 cells in G₀/G₁ phase was 53.42±2.14, 67.37±2.43 and 79.78±2.86%, respectively, which was significantly higher compared with the control group (38.68±1.96%; all P<0.05), demonstrating that paeonol dose-dependently induced a significant accumulation of HCT116 cells in G₀/G₁ phase. Furthermore, the cell cycle profile of HCT116 cells exposed

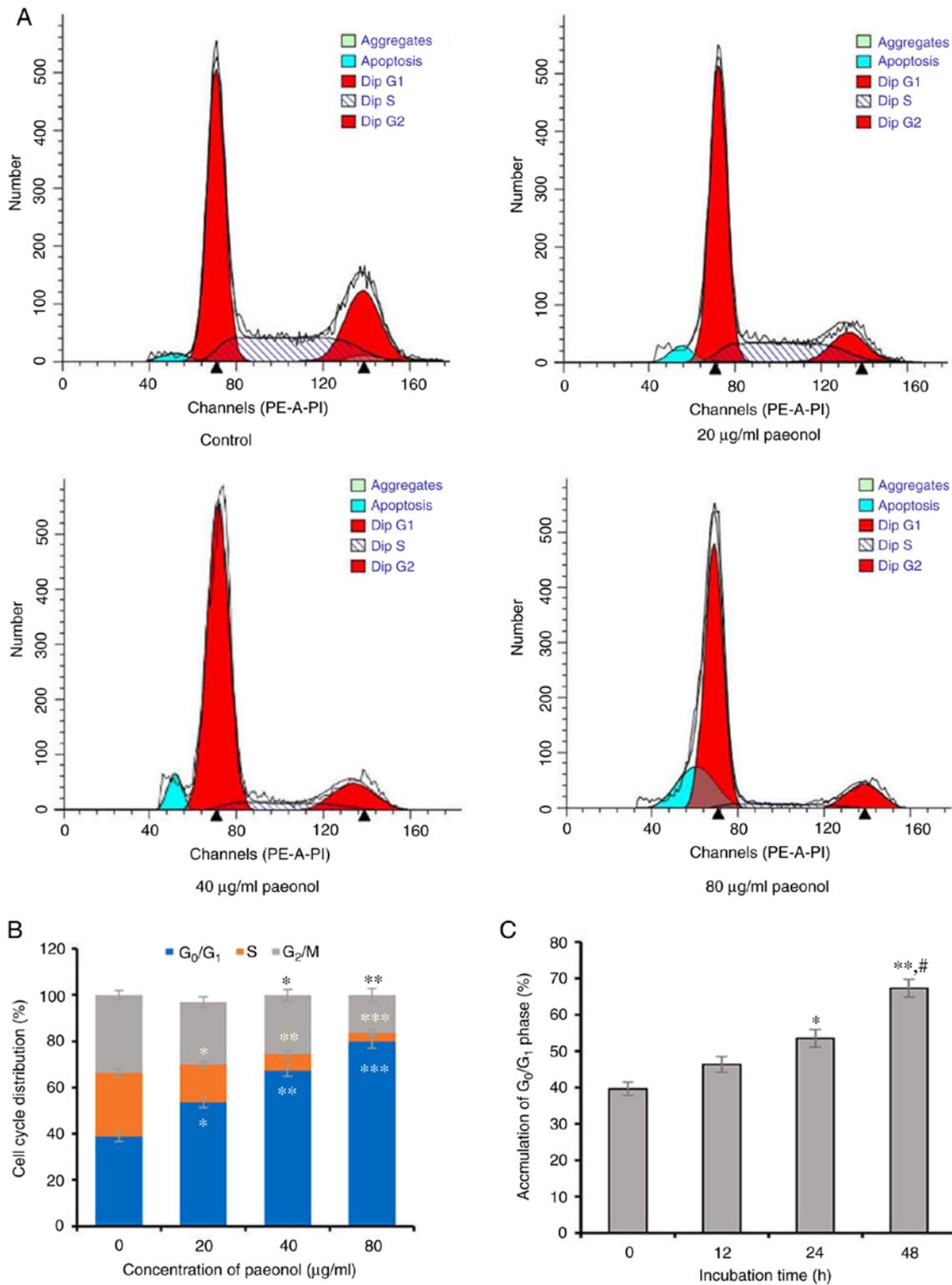


Figure 2. Effect of paeonol causes cell cycle arrest in HCT116 cells. (A) After incubation with different doses of paeonol for 48 h, the cellular DNA content was analyzed using flow cytometry. (B) Histogram of cell cycle distribution in HCT116 cells exposed to different doses of paeonol for 48 h. (C) G₀/G₁ phase accumulation of HCT116 cells exposed to 40 µg/ml paeonol for various durations. *P<0.05, **P<0.01 and ***P<0.001 vs. control group (0 µg/ml); #P<0.05 vs. the groups at the time point of 12 and 24 h. PI, propidium iodide.

to different doses of paeonol exhibited a distinctive broad sub-diploid DNA (sub-G₁) peak at 48 h, which was significantly different compared with the control cells (Fig. 2A). The accumulation of HCT116 cells in G₀/G₁ phase was also accompanied by corresponding decreased percentages in the S and

G₂/M phases (Fig. 2A and B). In addition, the proportion of HCT116 cells in G₀/G₁ phase was time-dependently increased in the presence of paeonol (Fig. 2C), thus suggesting that paeonol dose- and time-dependently inhibited the proliferation of HCT116 cells by causing G₀/G₁-phase arrest.

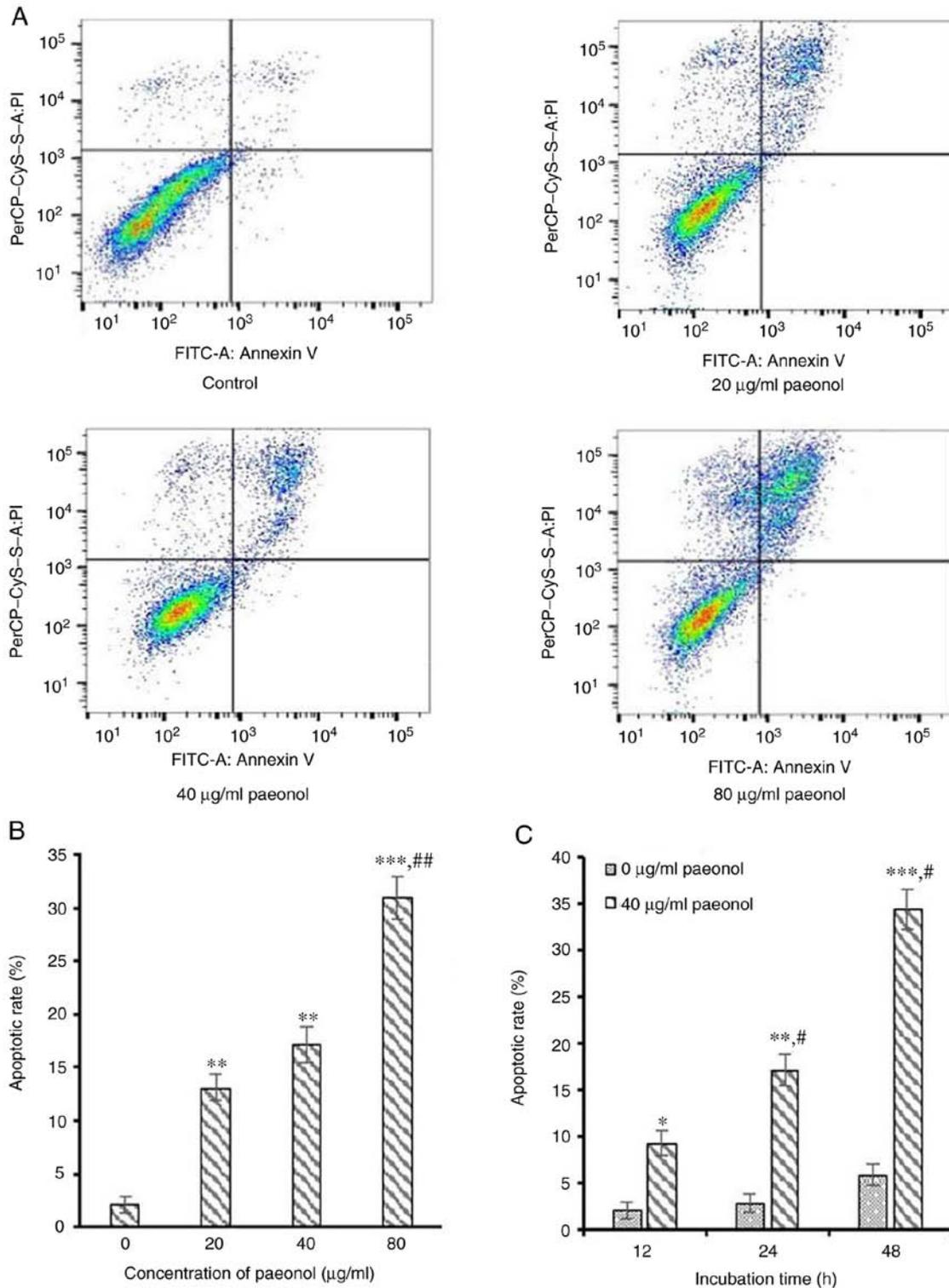


Figure 3. Effect of paeonol on the apoptotic rate in HCT116 cells. (A) After incubation with different doses of paeonol for 24 h, the apoptotic rate was measured using flow cytometry. (B) Histogram of apoptotic cells following exposure to different doses of paeonol for 24 h. (C) Histogram of apoptotic cells following exposure to 40 µg/ml paeonol for 12, 24 and 48 h. *P<0.05, **P<0.01 and ***P<0.001 vs. control group (0 µg/ml); #P<0.05 vs. the group at the time point of 12 h; and ##P<0.01 vs. 20 and 40 µg/ml paeonol groups or the groups at the time point of 12 and 24 h. PI, propidium iodide.

Paeonol induces apoptosis in HCT116 cells. To assess whether cell apoptosis was responsible for the reduction of viable cells following incubation with paeonol, an Annexin V-FITC/PI double staining assay with FCM analysis was used to monitor phosphatidylserine exposure. Following incubation with 20, 40 and 80 µg/ml paeonol for 24 h, the FCM results indicated that

the apoptotic rate of HCT116 cells was 13.07±1.23, 17.13±1.65 and 30.97±2.01%, respectively, which was significantly higher compared with the control group 4.20±0.83% (all P<0.05; Fig. 3A and B), indicating that paeonol dose-dependently induced apoptosis of CRC cells. Moreover, the apoptotic rates increased with longer exposure time of paeonol (Fig. 3C).

Therefore, the results indicated that paeonol dose- and time-dependently promoted apoptosis of CRC cells, which may be one of the underlying mechanisms of the anti-cancer activity of paeonol.

Paeonol induces cell apoptosis via the caspase-dependent pathway. Following incubation with increasing concentrations of paeonol for 48 h, caspase-3 activity gradually increased in HCT116 cells and was higher compared with the control group ($P < 0.05$). In addition, similar trends for caspase-8 and caspase-9 were observed in HCT116 cells exposed to paeonol for 48 h (Fig. 4). Collectively, it was demonstrated that paeonol dose-dependently increased the activity of caspase-3, -8 and -9.

Paeonol represses the β -catenin-mediated transcriptional activity of TCF/LEF. To examine the effect of paeonol on the activity of TCF/LEF mediated by β -catenin, a TCF/LEF luciferase reporter assay was performed. With increasing concentrations of paeonol, the luciferase activity gradually decreased and there was a significant difference between the 20 $\mu\text{g/ml}$ paeonol-treated group and the control group ($P < 0.05$). Furthermore, the luciferase activity following exposure to 80 $\mu\text{g/ml}$ paeonol was significantly weaker compared with the group treated with 20 $\mu\text{g/ml}$ paeonol ($P < 0.05$; Fig. 5), suggesting that a high concentration of paeonol significantly repressed the transcriptional activity of TCF/LEF.

Paeonol inhibits proliferation via the Wnt/ β -catenin signaling pathway. To elucidate the possible mechanism responsible for the inhibition of transition to the DNA synthesis phase, the cell cycle-associated proteins, including cyclin D1, CDK4 and p21^{Cip1}, which are able to promote cell cycle progression (23-24), were further investigated by western blot analysis. The protein expression levels of cyclin D1 and CDK4 were significantly downregulated, while p21^{Cip1} expression was upregulated in HCT116 cells exposed to paeonol for 48 h (Fig. 6). Thus, paeonol may be able to cause G₀/G₁ phase arrest at least partly due to interference of the expression levels of the key G₁-regulatory proteins CDK4, cyclin D1 and p21^{Cip1}.

To investigate the mechanisms underlying the anti-tumor effect of paeonol against HCT116 cells, which were via inducing cell apoptosis, western blot analysis was used to detect the expression levels of apoptosis-associated proteins. Following incubation with 20, 40 and 80 $\mu\text{g/ml}$ paeonol for 48 h, the expression levels of Bax and cytochrome C were significantly upregulated, while those of Bcl-2 were significantly downregulated in HCT116 cells in a dose-dependent manner (Fig. 6). Moreover, the Bax/Bcl-2 ratio was elevated compared with the control group and was dose-dependently increased by paeonol in HCT116 cells ($P < 0.05$). Collectively, the results indicated that paeonol induced cell apoptosis via increasing the Bax/Bcl-2 ratio in HCT116 cells.

To further elucidate whether paeonol exerts its anti-tumor effect against HCT116 cells via the Wnt/ β -catenin pathway, the protein expression levels of β -catenin, as well as its downstream signaling molecules cyclin D1, c-Myc and survivin proto-oncogene, were determined by western blot analysis. Following incubation with 20, 40 and 80 $\mu\text{g/ml}$ paeonol for 48 h, the expression levels of β -catenin, c-Myc and survivin were dose-dependently downregulated, while those of GSK-3 β

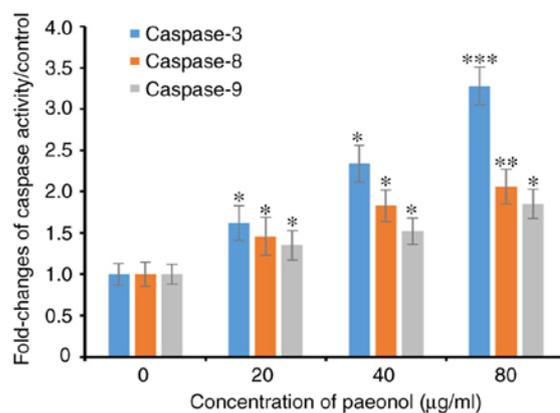


Figure 4. Effect of paeonol on the caspase activity in HCT116 cells. After incubation with various concentrations of paeonol for 48 h, the activities of caspase-3, -8 and -9 were determined using a colorimetric caspase assay. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control group (0 $\mu\text{g/ml}$).

were dose-dependently upregulated in HCT116 cells compared with the control group ($P < 0.05$; Fig. 6). In addition, paeonol significantly inhibited the expression of cyclin D1 compared with the control group ($P < 0.05$). Moreover, a possible mechanism responsible for the effects of paeonol against human CRC cells is schematically presented in Fig. 7.

Discussion

Despite progress in systemic anti-cancer therapy, the effective treatment of CRC remains a major clinical challenge due to its high mortality and metastasis potential (25). Active components, including hydnoicarpin (15), aesculetin (16), baicalin (17), isobavachalcone (18), wogonin (20) and lycorine (26), of Traditional Chinese Medicine formulations have attracted increased attention worldwide due to their unique advantages over western drugs in cancer treatment (26). For instance, experimental data have revealed that paeonol has mild anti-tumor activities (10,27,28). In the present study, paeonol time- and dose-dependently suppressed the viability of HCT116 cells, with an IC₅₀ of 79.60 $\mu\text{g/ml}$ at 48 h; these results are consistent with other CRC cell lines (LoVo and SW620) (29). Moreover, paeonol has been shown to be a relatively safe medicine in mice with a median lethal dose of 3,430 mg/kg (30). Collectively, these experimental data suggest that paeonol may be a novel candidate for anti-cancer therapy.

Cell proliferation is closely regulated by the cell cycle (22), and disruption of the cell cycle may inhibit cell proliferation and suppress tumor growth. The present results indicated that paeonol induced an accumulation in the G₀/G₁ phase, accompanied by a concomitant decrease in S and G₂/M phases in HCT116 cells, which is consistent with findings in other cancer cell lines (11,31). Cell cycle control is regulated by CDKs, cyclins and CDK inhibitors, such as p21^{Cip1} and p27^{Kip1} (23), and the activity of cyclin-CDK complexes may be affected by multiple signaling pathways (32). Furthermore, binding of cyclin D1 to CDK4 or CDK6 causes the formation of the cyclin D1-CDK4/6 complex, eventually driving cell transition from G₀/G₁ to S phase (33). In the present study, the expression levels of cyclin D1 and CDK4 were downregulated and that of p21^{Cip1} was upregulated, subsequently blocking the cell cycle progression. Thus, it was hypothesized that

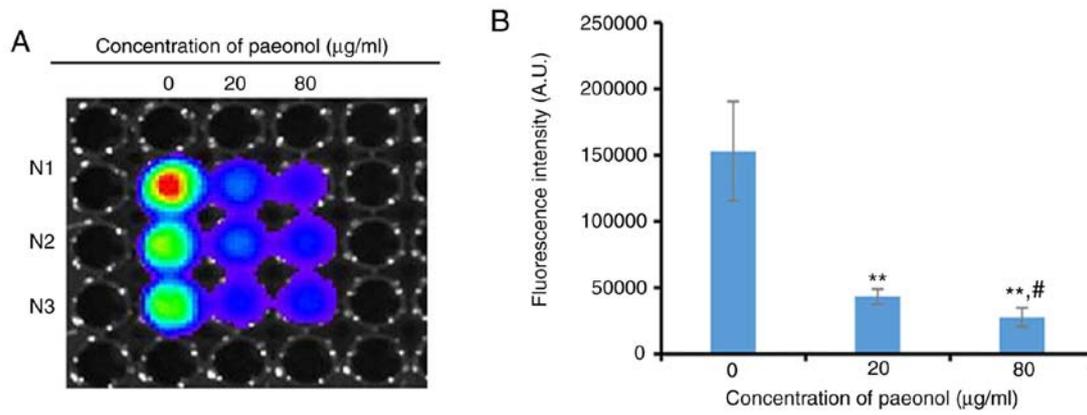


Figure 5. Effect of paeonol to interfere with T cell-specific transcription factor/lymphoid-enhanced factor activities. (A) Images of transfected HCT116 cells exposed to 0, 20 and 80 µg/ml paeonol in a 96-well dish were captured using the IVIS® Spectrum system (version 4.5 software). N1, N2 and N3 represent three experimental repeats. (B) Luciferase activities in the HCT116 cells exposed to various doses of paeonol for 48 h. **P<0.01 vs. control group (0 µg/ml); #P<0.05 vs. 20 µg/ml paeonol group. A.U., absorption units.

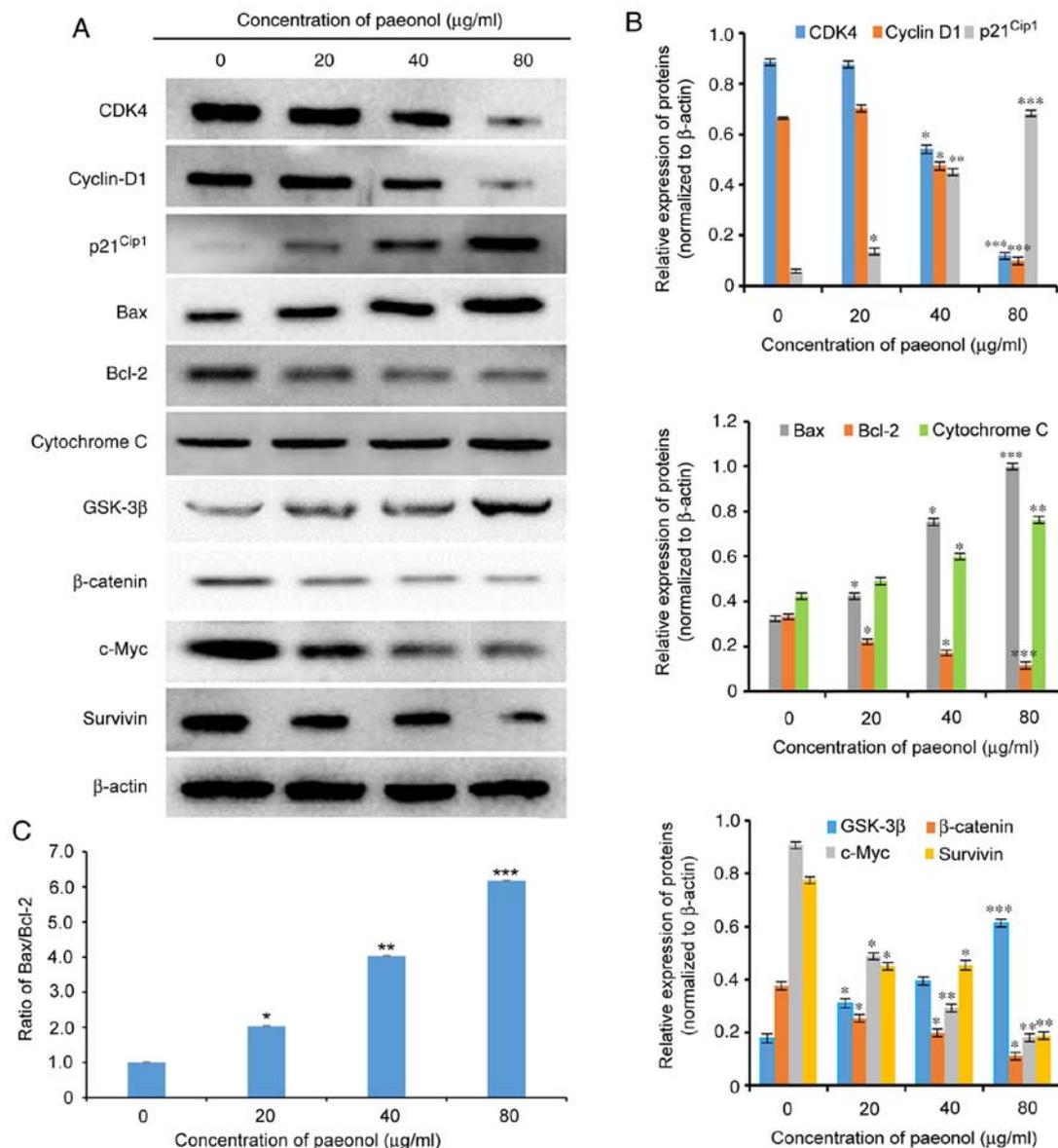


Figure 6. Effect of paeonol on the protein expression in HCT116 cells. (A) After incubation with different doses of paeonol for 48 h, the protein expression was detected by western blot analysis. (B) Quantification of cell cycle-coordinating proteins, apoptotic-associated proteins and GSK-3β, β-catenin, c-Myc and survivin proteins following exposure to different doses of paeonol for 48 h. (C) Ratio of Bax/Bcl-2. *P<0.05, **P<0.01 and ***P<0.001 vs. control group (0 µg/ml). GSK, glycogen synthase kinase; CDK, cyclin-dependent kinase.

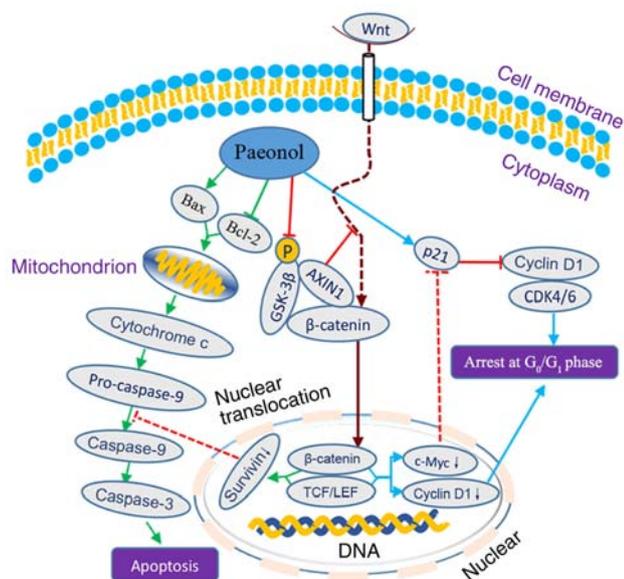


Figure 7. Schematic illustration of possible mechanisms responsible for the effects of paeonol against human colorectal cancer HCT116 cells. Arrows represent promotion; red straight line represents inhibition; red dot line represents a decrease of inhibition. TCF/LEF, T cell-specific transcription factor/lymphoid-enhanced factor; GSK, glycogen synthase kinase; CDK, cyclin-dependent kinase; P, phosphate; AXIN1, axin 1.

paeonol exerted anti-proliferative effects by blocking cell cycle transition from G₁ phase to S phase.

Induction of apoptosis is one of the most important and direct pathways that controls and eliminates cancer proliferation (11,34). The FCM results of the present study demonstrated that paeonol dose- and time-dependently induced cell apoptosis, which was in line with previous studies of CRC cells (29) and other cancer cells (11,31). In addition, HCT116 cells exposed to paeonol exhibited a distinctive broad sub-G₁ peak at 48 h, the appearance of which is usually regarded as a result of the degradation of nuclear DNA in the early stages of cell apoptosis (35). Although the mechanisms of apoptosis are complex, it has been reported that mitochondrial (intrinsic) and cell-surface death receptor-mediated (extrinsic) apoptosis are the two principal pathways (36). Bcl-2 family proteins have a significant role in the intrinsic apoptotic pathway, during which the imbalance between pro- and anti-apoptotic proteins determines the ultimate fate of cancer cells (37). Bax causes mitochondrial disruption, release of cytochrome c, activation of the downstream caspase-9 and, ultimately, activation of caspase-3 (38). The present results demonstrated an increase in the Bax/Bcl-2 ratio, via which the apoptotic effect of paeonol on HCT116 cells was exerted. To investigate which pathway was responsible for cell apoptosis, the activities of caspase-3, -8 and -9 were detected using a colorimetric caspase assay. Following incubation for 48 h, caspase-3, -8 and -9 activities in HCT116 cells were enhanced with increasing doses of paeonol in comparison with those in the control group. All of these results are consistent to a previous study, which showed that paeonol induces cell apoptosis via suppressing the expression of Bcl-2 and increasing the expression levels of Bax, caspase-8 and caspase-3 (39). Furthermore, the aforementioned results suggested that the suppressive effect of paeonol on the viability of CRC cells was associated with the induction of apoptosis via the caspase pathway.

In most healthy cells, the Wnt pathway is commonly inactive and β-catenin is sequestered in the cytoplasm by a multi-protein complex containing axin 1 (AXIN1), APC regulator of Wnt signaling pathway, casein kinase 1 α and GSK-3β (40), resulting in a low level of β-catenin in the nucleus. Activated Wnt may cause the translocation of β-catenin from the cell cytoplasm to the nucleus, where it activates the β-catenin-mediated LEF/TCF transcriptional machinery, thus inducing the transcription of TCF/LEF-responsive genes, such as c-Myc (41) and cyclin D1 (42). Moreover, abnormal activation of the Wnt signaling pathway, a known hallmark of CRC, has been reported to be associated with cell proliferation, cell cycle and cell apoptosis, as a result of an activated canonical β-catenin and LEF/TCF pathway (43), in which suppressing the expression of β-catenin has a beneficial anti-tumor effect (44). In the present study, the expression level of β-catenin was significantly downregulated, while that of GSK-3β was upregulated by paeonol in a dose-dependent manner.

c-Myc, a downstream effector of the β-catenin pathway, has been revealed to be upregulated in ~30% of cancer cells and is associated with cancer progression (45). With the disruption of β-catenin/TCF activity, decreased c-Myc may cause the transcription of p21^{Cip1/Waf1}, which in turn promotes cell cycle arrest at the G₀/G₁ phase and cell differentiation (46). Furthermore, cyclin D1, another downstream effector of the β-catenin pathway, exerts a vital role in regulating the cell cycle progression in different types of cells (47,48). The activated β-catenin signaling pathway may also stimulate the transcription of cyclin D1, which is an important marker for cells undergoing mitosis (49). The present findings identified the roles of c-Myc and cyclin D1 in cell cycle regulation, as demonstrated by the decreased TCF/LEF activity and concomitant downregulation of c-Myc and cyclin D1 protein. Apart from c-Myc and cyclin D1, survivin, an inhibitor of apoptosis, was identified as another target gene that is implicated in suppressing cell proliferation and regulating the cell life span (50). A previous study reported that downregulation of c-Myc, cyclin D1 and survivin may be an effective treatment strategy for CRC (51). Induction of TCF target gene transcription via activating the β-catenin pathway in CRC constitutes the primary transforming event, while TCF transcriptional activity may be reduced via suppressing the expression of β-catenin, subsequently followed by cell apoptosis via caspase-3 activation (52). The present results are in line with a previous study, reporting that blocking the interaction between β-catenin and TCF induced pancreatic cancer cell apoptosis via decreasing the expression levels of c-Myc and cyclin D1 (52). In addition, antagonism of Wnt/β-catenin signaling occurs at 20, 40 and 80 μg/ml paeonol to those required to suppress cell proliferation, block the cell cycle at G₀/G₁ phase and induce apoptosis in HCT116 cells. A schematic illustration of the possible mechanism underlying the anti-tumor activity of paeonol against CRC cells, including the induction of G₀/G₁ phase arrest and apoptosis via suppressing the Wnt/β-catenin signaling pathway is presented in Fig. 7. However, the lack of multiple cell lines to assess the present findings is a limitation of the current study. In addition, the suppressive effect of AXIN1 on Wnt signaling pathways and the intervention of Wnt/β-catenin signaling pathways on the cell cycle and apoptosis require further investigation in future studies.

In conclusion, the present results indicated that paeonol exerted an anti-tumor effect against CRC cells, which, at least partly, involved the blockage of the Wnt/ β -catenin signaling pathway. Therefore, the current findings support the use of paeonol as a novel treatment for CRC, acting via distinct mechanisms. However, in subsequent studies, the results of the present study should be verified using multiple cell lines and the long-term effects of paeonol are required to be assessed *in vivo*.

Acknowledgements

Not applicable.

Funding

This study was financially supported by Jiangsu Provincial Administration of Traditional Chinese Medicine (grant no. YB2017099).

Availability of data and materials

All data generated or analyzed during the present study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

LHL and RJS contributed to the conception and design of the study. LHL and ZCC performed the experiments and contributed to data analysis. LHL drafted the manuscript and RJS revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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