Research on the effect of ginseng polysaccharide on apoptosis and cell cycle of human leukemia cell line K562 and its molecular mechanisms

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Abstract. Ginseng polysaccharide (GPS), a polymer of glucose and the primary constituent extracted from panax ginseng, has been documented to exert various pharmacological properties, including anti-tumor properties. To provide further insights into the anti-tumor functions of GPS, the present study was designed to investigate the effect of GPS on apoptosis and the cell cycle of human leukemia cell line K562 cells, and its underlying mechanisms. The results demonstrated that GPS could inhibit K562 cell proliferation and induce apoptosis *in vitro* in a concentration- and time-dependent manner. The transcription of P38 and c-Jun NH2-terminal kinase (JNK) mRNA were significantly augmented, while the transcription of extracellular signal-regulated kinase (ERK) mRNA were significantly reduced following treatment with GPS compared with the control group (all $P<0.05$). In addition, GPS treatment markedly suppressed the expression of phosphorylated (p)-ERK, nuclear factor (NF)-κB p65 and cyclin D1, and increased the synthesis of p-P38 and p-JNK protein expression, as evidenced by immunofluorescence and western blotting analyses. In conclusion, the results indicate that the GPS-mediated MAPK/NF-κB/cyclin D1 signaling pathway serves a crucial role in cell cycle arrest and apoptosis of K562 cells.

Introduction

Leukemia is a group of hematological malignancies characterized by abnormal proliferation, decreased apoptosis and blocked differentiation of hematopoietic stem/progenitor cells (1). To date, the removal or deactivation of cancerous cells is the primary strategy of treating leukemia, and this is achieved by methods such as chemotherapy or bone marrow transplantation (2,3). However, it has numbers of side effects and high toxicity. Therefore, apoptosis- and differentiation-inducing therapy seems to be a promising strategy for the treatment of leukemia, particularly in patients who cannot tolerate the intensity of chemotherapy and bone marrow transplantation. Thus, finding effective inducers that are free of general cytotoxicity and can be used to treat leukemia have clinical significance (4).

Traditional Chinese medicine (TCM) has been used in cancer treatment for a long time and its use is supported with scientific evidence (5). As a Chinese herbal medicine, panax ginseng is a very important TCM for 'invigorating qi'. The most effective ingredients of ginseng are the ginseng polysaccharide (GPS) that prevents and treats tumors. Previous studies have demonstrated that GPS could induce the inhibition of proliferation and differentiation of leukemia K562 cells *in vitro*. In addition, it has reported that ginseng induced apoptosis in human multiple myeloma cells (6). However, the effectiveness of GPS in a leukemia cell line remains to be explored and validated.

The human leukemia cell line K562 has been widely used for studies of leukemia, and is established from a patient with chronic myelogenous leukemia (7). In the present study, we adopted experimental hematology technologies to observe the effect of GPS on the inhibition of proliferation and induction of apoptosis in K562 cells and explore its underlying mechanisms.

Materials and methods

Drugs and reagents. GPS was purchased from Pude Pharmaceutical Co., Ltd. (Datong, China). Roswell Park Memorial Institute (RPMI)-1640 medium was obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Corporation (Hangzhou, China). Six-well plates and 96-well plates were provided by Costar (Cambridge, MA, USA). MTT related reagents were obtained from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Annexin V-propidium iodide (PI) apoptosis detection kit was purchased from Zhongshan Biological Co. (Beijing, China). Rabbit monoclonal antibodies against extracellular signal-regulated kinase (ERK; sc-292838), phosphorylated (p)-ERK (sc-136521), P38 (sc-4708), p-P38 (sc-101758), c-Jun NH2-terminal kinase (JNK;

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sc-571) and p-JNK (sc-135642) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit monoclonal antibodies against cyclin D1 (EPR2241) and NF-κB p65 (E379) were provided by Epitomics (Burlingame, CA, USA). Rabbit monoclonal antibody against β-actin (BA1039) was purchased from Wuhan Boster Biological Technology, Ltd., (Wuhan, China).

Cell culture. K562 cells were obtained from the Laboratory of the Faculty of Basic Medical Sciences, Chongqing Medical University (Chongqing, China). K562 cells were cultured in RPMI-1640 medium with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified 5% CO_2 atmosphere, and were passaged every three days. GPS was sterilized by filter that was dissolved by RPMI-1640. Each test was repeated three times.

MTT assay. Viability of K562 cells was determined by MTT assay. At 90% confluence, K562 cells were adjusted to a density of $7x10^8$ cells/l and divided into the blank control group and the GPS group. The latter was subdivided into different final concentration groups with 25, 50, 100, 200, 400, 600 and 800 mg/l GPS. Cells were cultured in 96-well plates with 200 μl culture medium for each well and incubated at 37˚C. After being cultured for 24, 48 and 72 h, 5 g/l MTT was added to each well and incubated at 37˚C for 4 h. Then, the cell suspension was centrifuged for 5 min at 1,200 x g and 4˚C and the supernatants were removed. The crystals that had formed were dissolved by adding 150 μl dimethyl sulfoxide to each well. After mixing, the absorbance of the cells was measured at 570 nm by a microplate reader. Experiments were performed in triplicate and the individual mean value was used for statistical analysis.

Measurement of cell cycle by flow cytometry (FCM). The control group was incubated in normal conditions and the GPS group was incubated with 400 mg/l GPS for 48 h. K562 cells were washed twice with 0.02 M PBS (pH 7.2) and fixed in 70% ethanol at 4˚C. Then, the fixative was removed, and RNase and PI was added for staining for 30 min. PI-stained samples were analyzed using an Influx flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Each sample consisted of $3x10^4$ cells. The data were analyzed by CellQuest software (BD Biosciences), and the ratio of each phase of the cell cycle was calculated.

Annexin V assay. The control group was cultured in normal conditions and the GPS group was cultured with 400 mg/l GPS for 48 h. K562 cells of each group were adjusted to a density of $1x10^6$ cells/ml. Then, 1 ml cell suspension was selected and centrifuged at 1,000 x g and 4˚C for 5 min. Then, the supernatants were removed, RNase was added, and samples were placed in a water bath at 37˚C for 1 h. Then, samples were placed in ice and incubated with 0.5 mg/l PI and Annexin V. Finally, the cell apoptosis rate was detected by FCM.

Wright's staining. At 90% confluence, K562 cells were adjusted to the density of $7x10^8$ cells/l and divided into the blank control group and the GPS group. The control group was incubated in normal conditions and the GPS group was incubated with 400 mg/l GPS. After being cultivated for 48 h respectively, cells of the two groups were smeared on slides and treated with Wright's stain. Then, the morphology of cells was observed in the two groups under light microscopy.

Transmission electron microscopy (TEM). The control group was incubated in normal condition and the GPS group was incubated with 400 mg/l GPS. After being cultivated for 48 h, fresh specimens of K562 cells were fixed by immersing them immediately in 2.5% glutaraldehyde fixative for 24 h. Semi-thin sections (60 nm) were obtained by using an ultra-microtome. Then, the changes in ultrastructure of K562 cells were identified under TEM.

Immunofluorescence and confocal laser scanning microscopy. The control group was cultured in normal conditions and the GPS group was cultured with 400 mg/l GPS for 48 h. K562 cells were centrifuged at 1,000 x *g* and 4˚C for 5 min and then placed on slides. Them, slides were fixed with 4% paraformaldehyde at 4˚C for 20 min and washed three times with PBS for 5 min each. After blocking with 0.5% bovine serum albumin (Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 30 min, slides were incubated with mouse anti-p-ERK, p-P38, p-JNK and rabbit anti-NF-κB p65 and cyclin D1 (all at 1:150) overnight at 4˚C. Then, slides were washed three times with PBS, and treated with fluorescein isothiocyanate goat anti-mouse (1:100; BA1105) or rabbit (1:100; BA1101; both Wuhan Boster Biological Technology, Ltd.) IgG for 40 min at room temperature in the dark. After being mixed with PI for 1 min, slides were again rinsed with PBS three times, mounted with 50% glycerol and stored in the dark. Immunofluorescence was examined with a Leica Sp2 confocal microscope.

Western blot analysis. Expression of ERK, p-ERK, P38, p-P38, JNK, p-JNK, NF-κB p65 and cyclin D1 proteins was measured by western blotting. K562 cells were divided into the blank control group and the experimental group. In the experimental group, K562 cells were treated with 400 mg/l GPS at 37˚C for 6, 18, 24, 48 and 72 h. Briefly, cells were washed twice with PBS. Then, cell pellets were resuspended in lysate buffer, lysed on ice for 20 min, and then centrifuged at 12,000 x g at 4˚C for 15 min to remove nuclei and unbroken cells. Aliquots of supernatants containing 4 μg/μl of protein were suspended in SDS sample buffer and boiled for 5 min. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. The resulting gels were equilibrated in transfer buffer [25 mmol/l Tris-HCl, 192 mmol/l glycine and 20% methanol (pH 8.3)], and the proteins were transferred electrophoretically to polyvinylidene difluoride membranes and incubated for 1 h with PBS containing 5% skimmed milk and 0.05% Tween-20. Then, the membranes were incubated with antibodies against ERK, P38, JNK (all at 1:800), p-ERK, p-P38, p-JNK (all at 1:300), NF-κB p65 (1:3,000) and cyclin D1 (1:1,000) overnight at 4˚C. Specific biotinylated goat anti-rabbit antibodies (1:1,000; 7074P2; Cell Signalling Technologies, Inc., Danvers, MA, USA) were used to detect the primary antibodies for 1 h at room temperature. Antibodies against β-actin (1:500) were used for reference. Blots were developed with an ECL kit (WBKLS0100; EMD Millipore, Billerica, MA, USA)

according to the manufacturer's instructions. The results were analyzed by Photoshop version 6.0 software (Adobe Systems, Inc., San Jose, CA, USA).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from *K562* cell using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and quality was verified by resolving samples using 1% agarose gel electrophoresis. The following primers were used: ERK forward, 5'-CCCAAATGCTGACTCCAAAG-3', and reverse 5'-TCGGGTCGTAATACTGCTCC-3'; P38 forward 5'-ACC GTTTCAGTCCATCATTC-3', and reverse 5'-GTCAGCTTC TGGCACTTCAC-3'; JNK forward, 5'-CAAGCAGTTAGA TGAAAGGGAA-3', and reverse 5'-CAGACGACGATGATG ATGGA-3'; and GAPDH forward 5'-ACAGCCTCAAGATCA TCAGCA-3', and reverse 5'-TGAGTCCTTCCACGATAC CAA-3'. The PCR conditions were as follows: Denaturing at 94˚C for 5 min, 94˚C for 30 sec, 58˚C for 30 sec and 72˚C for 20 sec; target genes underwent 34 cycles and GAPDH underwent 28 cycles at 72˚C for 10 min. PCR products were resolved by using 2% agarose gel electrophoresis for 40 min. The results were observed under an ultraviolet lamp, photos were captured and the optical densities of bands were quantified and analyzed using UVI-Map V.99 software (UVItec, Ltd., Cambridge, UK).

Statistical analysis. The data are presented as the mean ± standard deviation. Data were analyzed using SPSS version 13.0 statistical package (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and Student's t-test were used with the Bonferroni method to determine statistically significant differences. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Growth inhibition and cell cycle perturbation were induced by GPS. The results indicated that exposure of K562 cells to GPS at different concentrations (25, 50, 100, 200, 400, 600 and 800 mg/l) for 24, 48 and 72 h caused significant inhibition to the proliferation of cells in a dose-dependent manner ($P=0.03$; Fig. 1A). Exposure to 400 mg/l GPS for 48 h resulted in IC_{50}. Therefore, 400 mg/l GPS treatment for 48 h was used in the following experiments of K562 cells. In addition, it was identified that the G0/G1 phase of the cell cycle increased significantly ($P=0.02$), while the G2+M and S phases of the cell cycle decreased significantly ($P=0.02$) after GPS (400 mg/l) treatment, in a time-dependent manner (Fig. 1B). These results demonstrated that GPS could inhibit K562 cell proliferation and arrest the cell cycle in the G0/G1 phase.

GPS triggers apoptosis in K562 cells. The percentage of apoptotic cells following treatment with GPS at 400 mg/l for 24, 48 and 72 h in K562 cells were analyzed by flow cytometry. Results of Annexin V/PI double staining confirmed that after adding 400 mg/l GPS for 24 (23.00±0.65%), 48 (43.85±0.87%) and 72 h (49.56±0.93%) the apoptosis rates of K562 cells were significantly increased compared with the control group (3.82±0.23%; $P<0.05$; Fig. 2). Furthermore, TEM was used to observe the changes in ultrastructure morphology in K562 cells upon GPS treatment (400 mg/l for 48 h). The results showed that increased blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and the formation of apoptotic bodies were present in GPS-treated K562 cells in comparison with untreated control cells (Fig. 3).

Morphological changes. In the control group, the nuclear volume was relatively high. The nucleoli were clearly visible and the nuclei:cytoplasm ratio was high. As can be observed in Fig. 4, the cytoplasm showed strong basophilia with specialized granules (Fig. 4A). Compared with the control group, the cell volume and nuclear diameter in the GPS group were smaller after 48 h (Fig. 4B; black arrows). The cytoplasm was abundant and the nuclei:cytoplasm ratio was decreased. Interestingly, in the GPS group a number of cells showed nuclear division, and the basophilia in the cytoplasm was weakened (Fig. 4B; red arrows).

GPS reduces the expression of p-ERK, NF-Bp65 and CyclinD1, while increase the expression of P-P38 and P-JNK in K562 cells. After treatment with 400 mg/l GPS for 48 h, the expression of p-ERK (Fig. 5), NF-κB p65 (Fig. 6) and cyclin D1 (Fig. 7) protein evidently decreased, while the expression of p-P38 (Fig. 8) and P-JNK (Fig. 9) protein increased, particularly the protein in the nucleus. Moreover, as shown in Fig. 6, the expression of NF-κB p65 decreased markedly and was transferred from the nucleus to the cytoplasm and cell membrane in K562 cells. After being incubated with 400 mg/l GPS for 0, 6, 18, 24, 48 and 72 h, it was observed that the variation of p-ERK, NF-κB p65, cyclin D1, p-P38 and p-JNK protein were expressed in a time-dependent manner. However, the expression of ERK, P38 and JNK did not change, as evidenced by western blotting analysis (Fig. 10).

GPS upregulated the transcription of P38 and JNK mRNA, and downregulated the transcription of ERK mRNA. After treatment with 400 mg/l GPS for 48 h, the transcription of ERK mRNA was significantly decreased ($P<0.05$), while the transcription of P38 and JNK mRNA were significantly increased ($P<0.05$; Fig. 11). In conclusion, these results indicate that GPS-mediated MAPK/NF-κB/Cyclin D1 signaling pathway played a crucial role in the cell cycle arrest and apoptosis of K562 cells.

Discussion

In the present study, human erythroleukemia cell line K562 cells were cultured from chronic myelogenous leukemia patients' pleural fluid. Since drugs can affect erythroid, granulocyte-monophyletic and megakaryocytic cell differentiation, it is an ideal model for the study of cell proliferation and differentiation, since its biological features are similar to the normal hematopoietic stem cell (CFU-Mix) (8). In the current study, the primary ingredient of ginseng, GPS, was demonstrated to inhibit proliferation and induce cell apoptosis of K562 cells. Furthermore, it was observed that GPS induces apoptosis and differentiation of K562 cells by influencing the MAPK signaling pathway.

Ras-MAPK signal transduction pathway is a type of serine/threonine protein kinase in cells, and serves a key

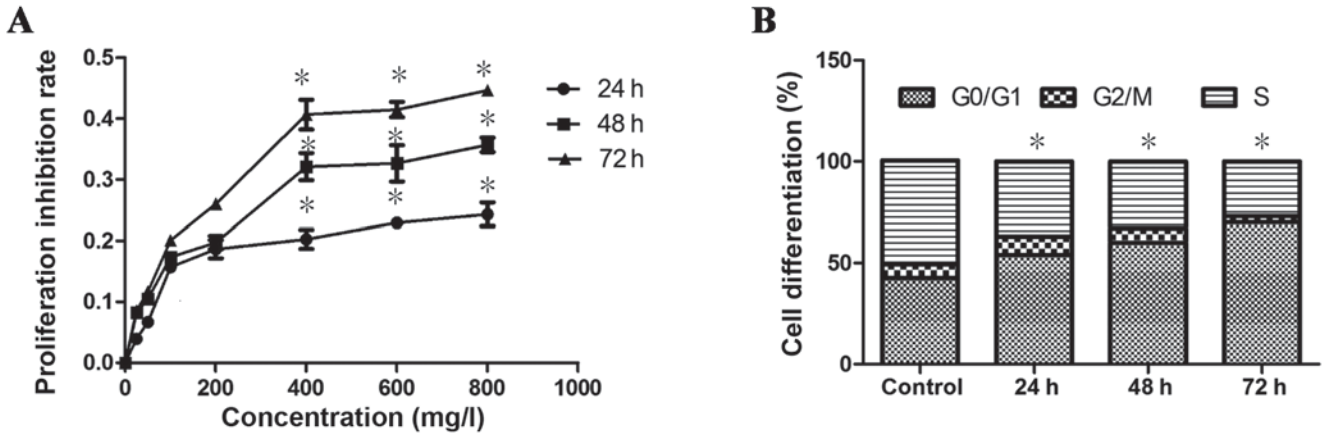

Figure 1. Effect of GPS on the cell cycle and proliferation of K562 cells. (A) Survival assay (MTT) was performed with the K562 cell line after treatment with GPS (20-800 mg/l) for 24, 48 and 72 h. *P<0.05 vs. untreated cells in suspension or in adhesion. Data are presented as the mean ± standard error (n=6). (B) K562 cells were treated with 400 mg/l GPS for 24, 48 and 72 h. *P<0.05 vs. the blank control group. Data are presented as the mean ± standard error (n=3). GPS, ginseng polysaccharide.

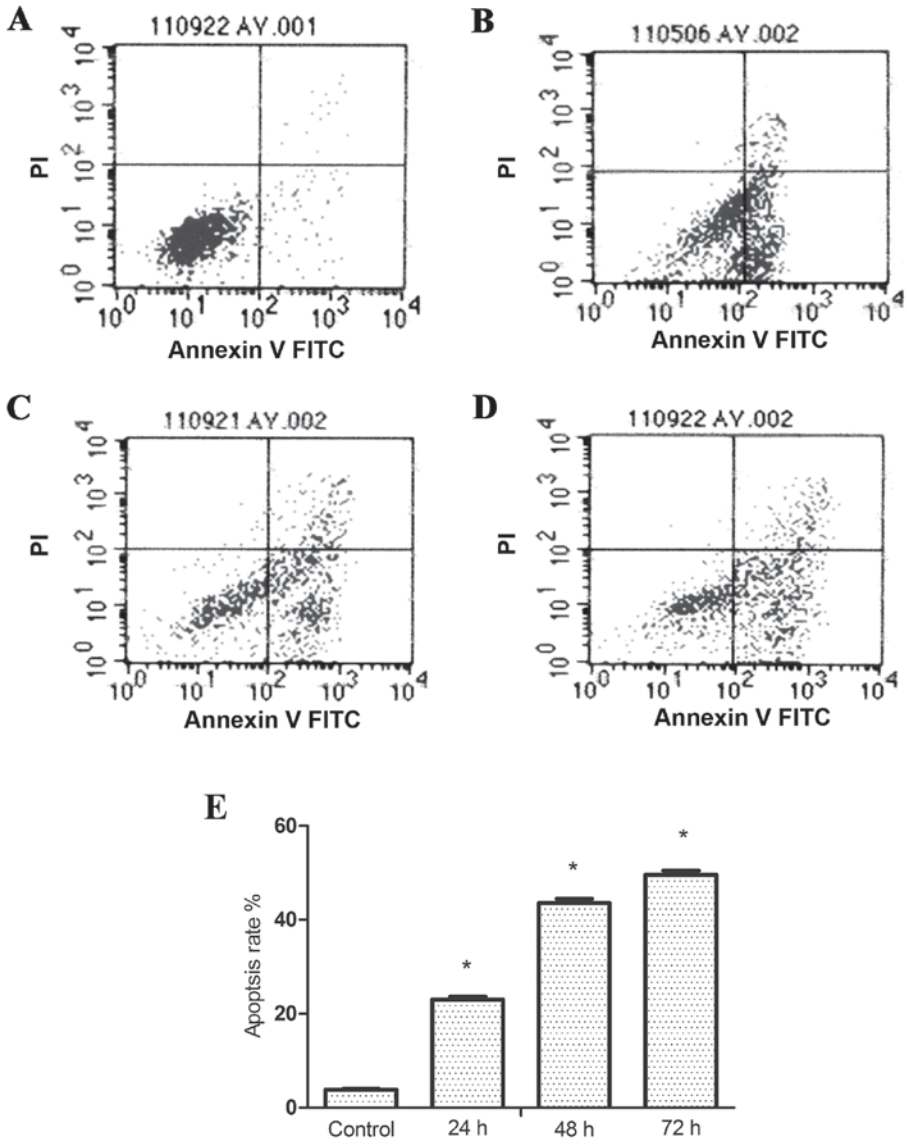

Figure 2. Effects of GPS on the apoptosis of K562 cells. The (A) control group and GPS groups treated with 400 mg/l GPS and incubated for (B) 24 (C) 48 and (D) 72 h were analyzed for apoptosis by Annexin V FITC and PI staining by flow cytometric analysis (E). Annexin V-, PI- cells are live cells, Annexin V+, PI- cells are early apoptotic cells, and Annexin V+, PI+ cells are late apoptotic cells. *P<0.05 compared with the control group. FITC, fluorescein isothiocyanate; GSP, ginseng polysaccharide; PI, propidium iodide.

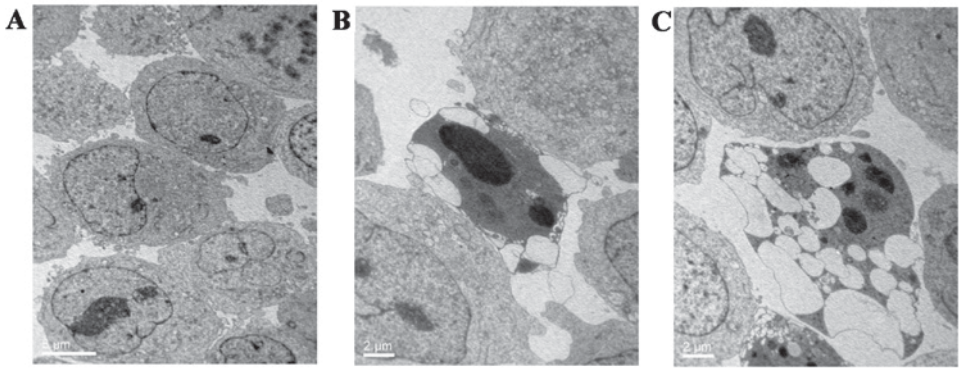

Figure 3. Ultrastructure of K562 cells by TEM. Representative images showing he changes in ultrastructure of K562 cells by TEM in (A) the control group at magnification, x3,500, or the GPS group at (B) magnification x5,000 and (C) x3,500. The control group were cultured in normal conditions and the GPS group were cultured with 400 mg/l GPS for 48 h. GPS, ginseng polysaccharide.

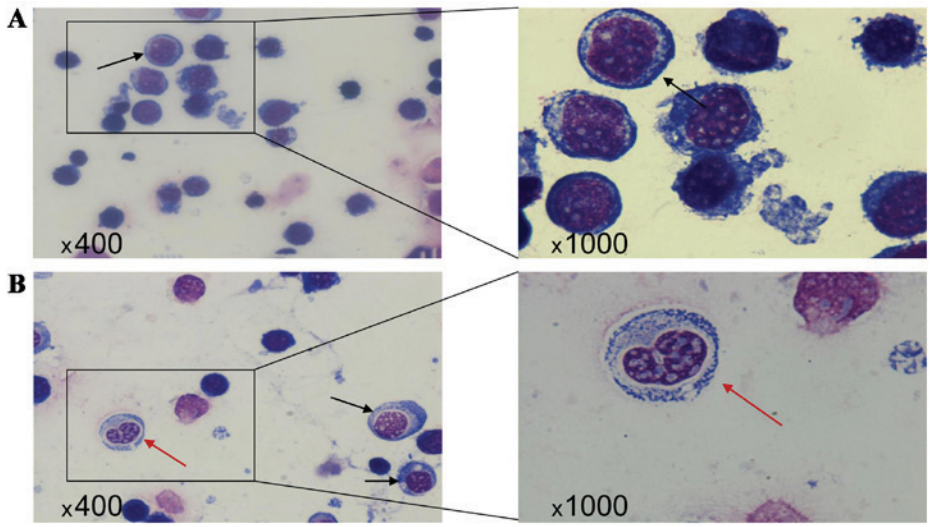

Figure 4. Effect of GPS on K562 cells observed by Wright's stain. (A) K562 cells in the control group. The black arrows indicate that the cell volume and nuclear diameter in the GPS group were smaller after 48 h. (B) K562 cells treated with 400 mg/l GPS for 48 h. The red arrows indicate that, in the GPS group, a number of cells showed nuclear division, and the basophilia in the cytoplasm was weakened. Left half of the figure, x400 magnification; right half of the figure, x1,000 magnification.

role in the pathogenesis of chronic myeloid leukemia (9). The activation of MAPK by means of conservative 3-level kinase cascade, the kinase of MAPK kinase (MAPKKK, MAP3K, MEKK or MKKK) activates MAPK kinase (MAPKK, MAP2K, MEK or MKK), then MAPK kinase activates MAPK, and the performance of this activation reaction is through successive phosphorylation (10). The activated MAPK participates in a number of cell responses, such as maintaining cell survival and inducing apoptosis (11). The MAPK family primarily includes three groups, including ERK1/2, P38 mitogen-activated protein kinase, P38 and JNK. Among them, the ERK1/2's primary response is the stimulation of growth factors and the mediating of the cell proliferation signal (12). If it remains activated, it can cause cells to undergo malignant transformation. One study showed that the anti-apoptotic features of the K562 cell line associated with activity of ERK were more pronounced than in control cells, and that inhibition of the activity of ERK can cause K562 cell apoptosis (13). In addition, JNK and P38 primarily mediate cells apoptosis, differentiation and inflammation caused by emergency stimuli (14,15). This demonstrates that the MAPK system controls the cell proliferation, differentiation and apoptosis processes, and that a disruption of any one of them can cause excessive cell growth and result in cancer.

In previous studies, it was demonstrated that GPS can promote the proliferation and differentiation of pluripotent hemopoietic stem cells (CFU-Mix) (16-19). Meanwhile, increasing attention has been paid to the role of ginseng in cancer therapies. The primary strategy of the treatment for leukemia is to inhibit the proliferation of abnormal differentiated cells. In this study, different concentrations of GPS were used to examine K562 cells *in vitro*, and the effect of GPS was observed on the proliferation of K562 cells. MTT assay showed that GPS significantly inhibited the proliferation of K562 cells in a time- and dose-dependent manner. By analyzing the experimental results, it was concluded that GPS promoted normal hematopoiesis, and inhibited the proliferation of leukemic cells. It is hoped that the conclusion will provide theoretical basis and positive prospects for clinical applications.

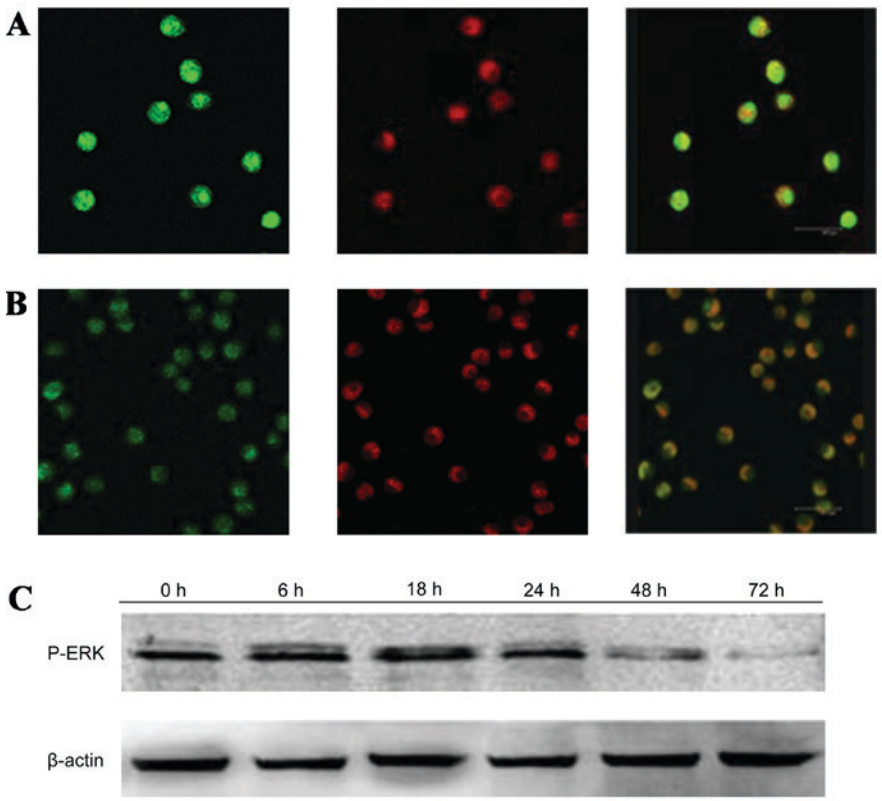

Figure 5. Effect of GPS on p-ERK protein expression in K562 cells. P-ERK was identified by immunofluorescence double staining in (A) the control group and (B) the GPS group, treated with 400 mg/l GPS. Green fluorescence, protein; red fluorescence, nucleus. (C) p-ERK protein analyzed by western blotting, following the treatment of cells with 400 mg/l GPS for different time periods. GPS, ginseng polysaccharide; p-ERK, phosphorylated extracellular signal-regulated kinase.

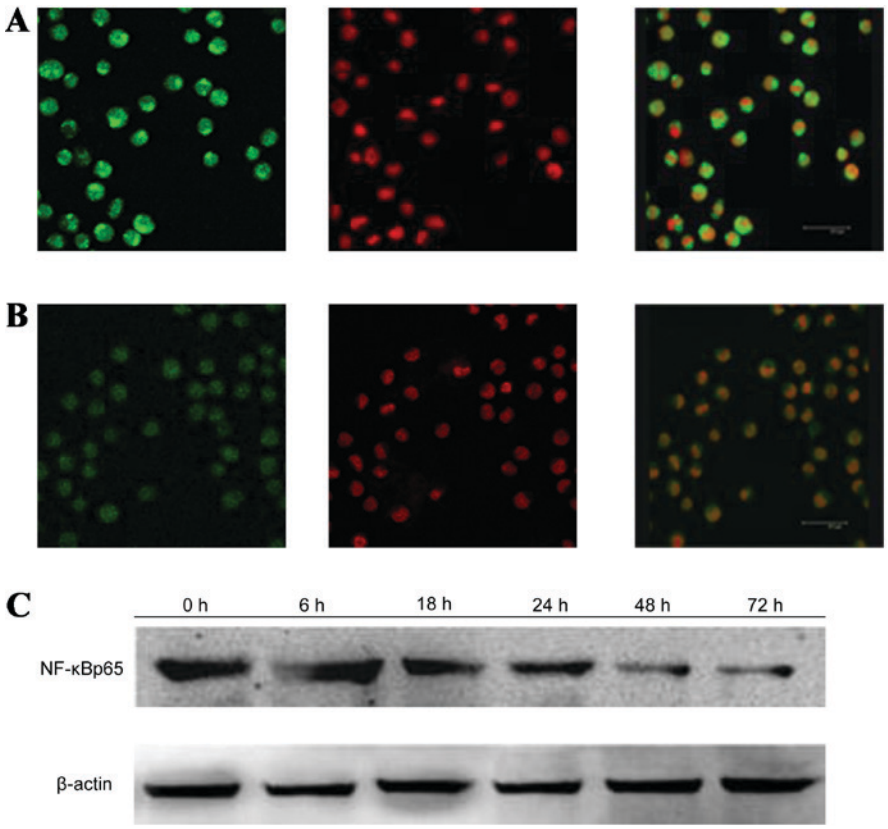

Figure 6. Effect of GPS on p-P38 protein expression in K562 cells. P-P38 was identified by immunofluorescence double staining in (A) the control group and (B) the GPS group, treated with 400 mg/l GPS. Green fluorescence, protein; red fluorescence, nucleus. (C) p-P38 protein analyzed by western blotting, following the treatment of cells with 400 mg/l GPS for different time periods. GPS, ginseng polysaccharide; p-P38, phosphorylated P38.

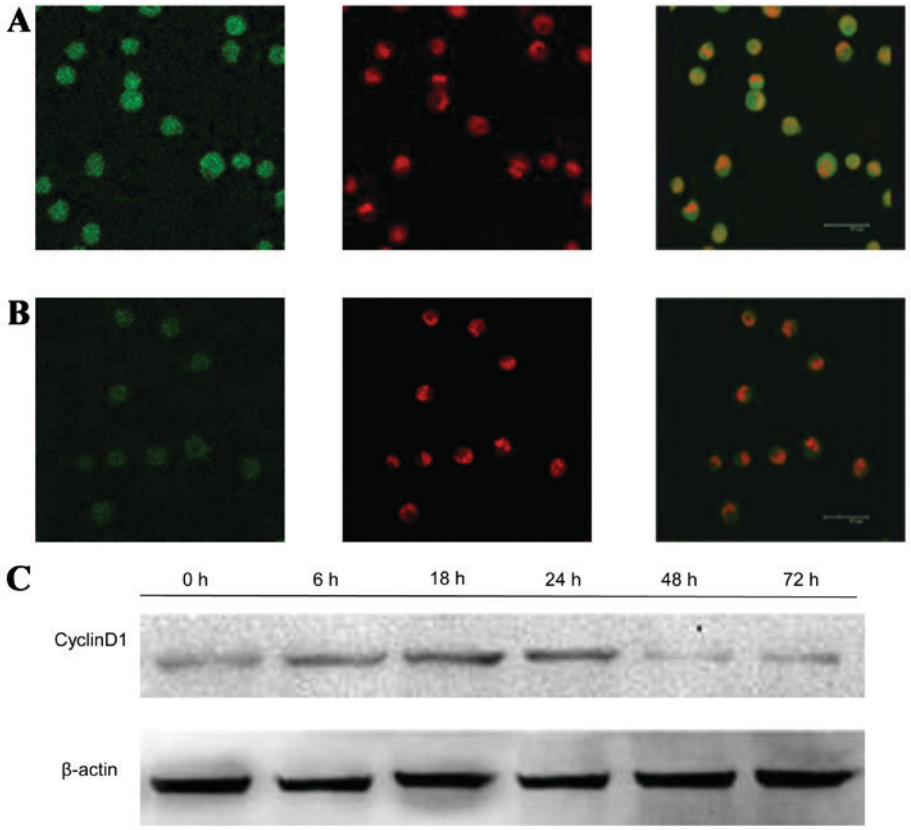

Figure 7. Effect of GPS on p-JNK protein expression in K562 cells. P-JNK was identified by immunofluorescence double staining in (A) the control group and (B) the GPS group, treated with 400 mg/l GPS. Green fluorescence, protein; red fluorescence, nucleus. (C) p-JNK protein analyzed by western blotting, following the treatment of cells with 400 mg/l GPS for different time periods. GPS, ginseng polysaccharide; p-JNK, phosphorylated c-Jun N-terminal kinase.

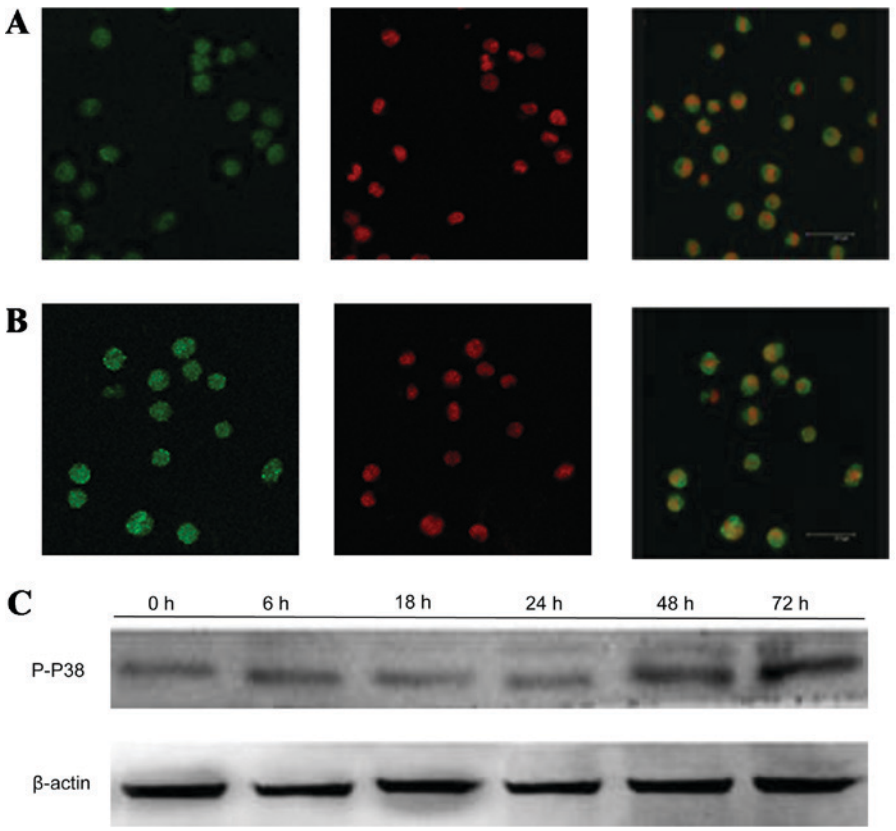

Figure 8. Effect of GPS on NF-κB p65 protein expression in K562 cells. NF-κB p65 was identified by immunofluorescence double staining in (A) the control group and (B) the GPS group, treated with 400 mg/l GPS. Green fluorescence, protein; red fluorescence, nucleus. (C) NF-κB p65 protein analyzed by western blotting, following the treatment of cells with 400 mg/l GPS for different time periods. GPS, ginseng polysaccharide; NF-κB p65, nuclear factor-κB p65.

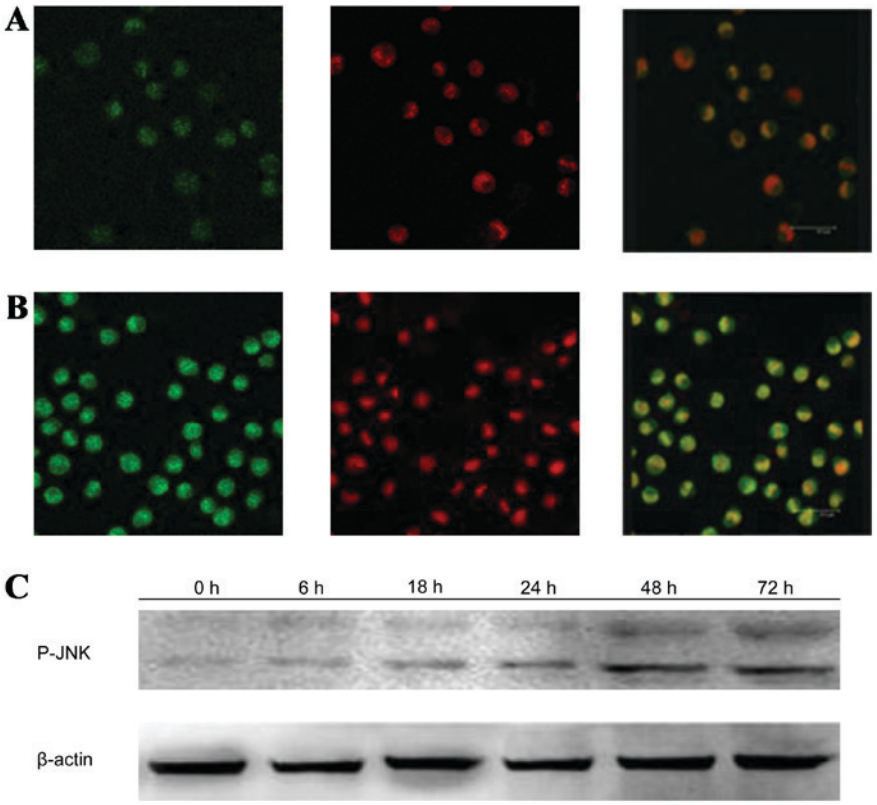

Figure 9. Effect of GPS on cyclin D1 protein expression in K562 cells. Cyclin D1 was identified by immunofluorescence double staining in (A) the control group and (B) the GPS group, treated with 400 mg/l GPS. Green fluorescence, protein; red fluorescence, nucleus. (C) Cyclin D1 protein analyzed by western blotting, following the treatment of cells with 400 mg/l GPS for different time periods. GPS, ginseng polysaccharide.

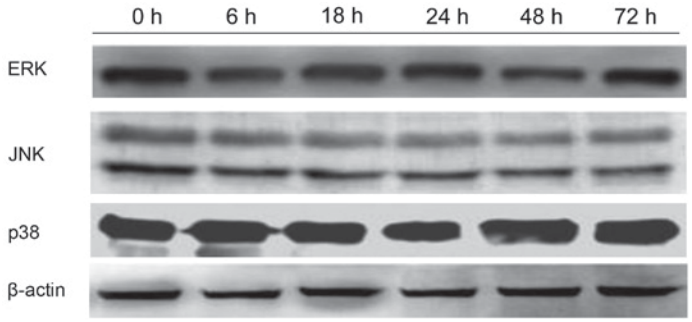

Figure 10. Effect of GPS on ERK, P38 and JNK protein expression in K562 cells by western blotting. The blots showed no obvious change in protein expression following treatment of K562 cells with GPS at 400 mg/l. ERK, extracellular signal-regulated kinase; GSP, ginseng polysaccharide; JNK, c-Jun N-terminal kinase.

Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes and death. Unlike necrosis, apoptosis produces cell fragments called apoptotic bodies that phagocytic cells are able to engulf and quickly remove before the contents of the cell can spill out onto surrounding cells and cause damage. Apoptosis is a multi-step, multi-pathway cell-death program that is inherent in every cell of the body. In cancer, the apoptosis cell division ratio is altered. Cancer treatment by chemotherapy and irradiation kills target cells primarily by inducing apoptosis. In the present study, a large number of apoptotic cells were observed by TEM in the 400 mg/l GPS group after 48 h. These changes included blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and the formation of apoptotic bodies. Therefore, it was demonstrated that GPS can induce apoptosis of K562 cells and arrest tumor progression.

MAPK signaling pathway regulates the survival, growth and apoptosis of cells (20). The abnormal activation of ERK in tumor cells can be observed. Therefore, blocking the ERK signaling pathway is an important method for cancer treatment. P38 and JNK serve a pivotal role in apoptosis (21). In the present study, results of immunofluorescence demonstrated that the protein expression of p-ERK was located in the nucleus and cytoplasm in the control group. Compared with the control group, expression of p-ERK protein, particularly protein in the nucleus, decreased markedly in the 400 mg/l GPS group at 48 h. The protein expression of p-P38 was located in the nucleus and cytoplasm in the control group.

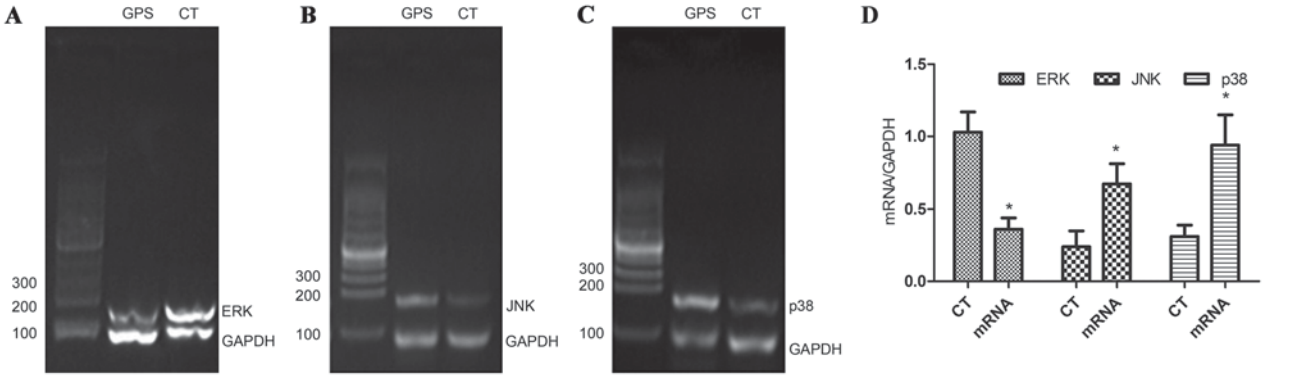

Figure 11. Effect of GPS on the expression of (A) ERK, (B) P38 and (C) JNK mRNA in K562 control cells, and K562 cells treated with 400 mg/l GPS for 48 h. mRNA was determined by semi-quantitative reverse transcription-polymerase chain reaction and normalized to GAPDH (D). Data are means ± standard error (n=3 each group). *P<0.05. ERK, Extracellular signal-regulated kinase; GSP, ginseng polysaccharide; JNK, c-Jun N-terminal kinase.

Compared with the control group, the protein expression of p-P38, particularly protein in the nucleus, increased markedly in the 400 mg/l GPS group at 48 h. The protein expression of p-JNK was located primarily in the cytoplasm in the control group. Compared with the control group, protein expression of p-JNK, particularly in the nucleus, increased significantly in the 400 mg/l GPS group at 48 h.

Results of RT-PCR confirmed that the expression of ERK mRNA in the 400 mg/l GPS group after 48 h was lower than that in the blank control group, while the expression of P38 and JNK mRNA was higher than that in the blank control group. Results of western blotting demonstrated that the expression of ERK, P38 and JNK has not changed markedly, while p-ERK decreased, and p-P38 and p-JNK were increased, compared with control group, following the treatment of cells with 400 mg/l GPS for different time periods. A number of studies have shown that activated MAPK, namely p-ERK, p-P38 and p-JNK, participate in the processes of gene transcription and the induction of apoptosis (20,22). Hence, the effect of GPS on the apoptosis of K562 cells may be caused by the phosphorylation of MAPK and the activation of downstream pro-apoptotic proteins, followed by changes in the location of p-ERK, p-P38 and p-JNK.

The NF-κB signaling pathway is a key pathway in the occurrence and progression of tumors. Anomalous activation of the NF-κB signaling pathway can lead to abnormal expression of a wide range of tumor-associated genes, regulation of the proliferation and apoptosis of cells, transformation of normal cells, and the formation and transfer of tumor vessels, which have direct impacts on the occurrence and progression of malignancies (23). Therefore, NF-κB may be a key target for the prevention of human cancer. Baldwin (24) demonstrated that MAPK and NF-κB signaling pathways are closely associated, and that the activation of NF-κB is indispensable for the oncogenes BCR-ABL and Ras transforming. Each molecule in the MAPK signaling pathway serves an important role in the activation of NF-κB. Studies have shown that phosphorylation of ERK can promote the phosphorylation of IK kinase (IKK), which promotes the activation of NF-κB; this is called the ERK-IKKs-IκB-NF-κB signaling cascade (25,26). The rapid and sustained activation of P38 caused by vitamin C inhibits the activation of IKK caused by tumor necrosis factor (TNF), while the use of a P38 inhibitor causes the increase of NF-κB (27). Baldwin (24) showed that the inactivation of cyclin D1/cyclin-dependent kinase 4 caused by P38 reduces the nuclear translocation of NF-κB in colorectal cancer. The inhibition of P38, JNK and NF-κB signaling pathways caused by disulfiram inhibits the performance of breast cancer stem cells (28). Baud and Karin (29) discovered that NF-κB and JNK signaling pathways are negatively associated. In gliomas, the inhibition of matrix metalloproteinase 2 reduces the activation of NF-κB mediated by TNF-α, and then causes the cell death mediated by JNK (30). The inhibition of NF-κB requires the activation of JNK, which is one of the mechanisms for apoptosis (31-33). A number of researchers have maintained that one of the possible mechanisms of cell apoptosis mediated by JNK3 may be that the transcription of NF-κB is arrested, followed by the phosphorylation of p65 protein by JNK3 (34,35). In the present study, the expression of p-ERK and NF-κB decreased in a time-dependent manner, while p-P38 and p-JNK increased in a time-dependent manner in the 400 mg/l GPS group, which suggests that the apoptosis of K562 cells is caused by the inhibition of the MAPK/NF-κB signaling pathway. However, the question whether all of the molecules in the MAPK signaling pathway are involved in the process of NF-κB activation or not requires further investigation.

The cell cycle consists the G1, S, G2 and M phases. Cell cycle checkpoints are used by the cell to monitor and regulate the progress of the cycle. Checkpoints prevent cell cycle progression at specific points, allowing verification of necessary phase processes and repair of DNA damage. The cell is not able to proceed to the next phase until checkpoint requirements have been met. Several checkpoints are designed to ensure that damaged or incomplete DNA is not passed on to daughter cells. Two primary checkpoints exist, including the G1/S checkpoint and the G2/M checkpoint. G1/S transition is a restriction point and a rate-limiting step in the cell cycle. An alternative model of the cell cycle's response to DNA damage has previously been proposed, and it is known as the post-replication checkpoint. In this response, two key classes of regulatory molecules, cyclins and CDKs, determine a cell's progress through the cell cycle (36). Cyclin D1 has the function of regulating cells entering the G1 phase. When cycling D1 is expressed at low levels, cells are arrested in the G1 phase. Regulation of cell cycle is one of the most important biological processes in cells,

and disorders in the cycle can lead to malignancies. Cell cycle arrest may be a new target for the exploitation of novel drugs. However, the mechanism of effect of GPS on K562 cell cycle arrest remains unknown and further research is required. In the present study article, GPS was used to affect the growth of K562 cells, and it was discovered that the cell cycle was arrested in the G0/G1 phase, which was likely associated with the differentiation and apoptosis of leukemia cells.

Cyclin D1 is a member of the cyclin protein family that is involved in regulating cell cycle progression. The synthesis of cyclin D1 is initiated during the G1 phase, and its synthesis drives the G1/S phase transition. Overexpression of cyclin D1 enables persistent proliferation of cells and serves an important role in the process of carcinogenesis (37,38). In particular, overexpression of cyclin D1 can be found in a variety of malignant tumors. Polynucleotide chains of cyclin D1 are adopted in the treatment of lung cancer (39). It has been found that cyclin D1 and p53 are the downstream target genes of NF-κB. NF-κB regulates the expression of cyclin D1, and p53 arrests the cell cycle and inhibits the proliferation of tumor cells (40). Cyclin D1 promoter contains two binding sites for NF-κB. The activation of NF-κB has the function of promoting cyclin D1 expression and driving the G1/S phase, so that normal cells change to malignant cells (41). In this study, it was observed that NF-κB and cyclin D1 decreased in a time-dependent manner following treatment with GPS. It can be speculated that one of the probable mechanisms of the effect of GPS on K562 apoptosis may be that the inhibition of the MAPK/NF-κB-mediated signaling transduction pathway can inhibit the expression of cyclin D1, followed by the arrest if the cell cycle in the G0/G1 phase, which ultimately induces the apoptosis of K562 cells.

In conclusion, GPS can inhibit proliferation and induce apoptosis of K562 cells by arresting cell cycle at the G0/G1 phase. In addition, the results demonstrated that MAPK/NF-κB/cyclin D1 serves a crucial role in cell cycle arrest and the induction of apoptosis of K562 cells. Consequently, the MAPK/NF-κB/cyclin D1 signaling pathway is a potential molecular target for the treatment of leukemia and has promising prospects.

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