Original Article
Puerariae radix flavone induces cell cycle arrest and apoptosis of NB4 cells accompanied with JNK and mitochondrial pathway activation

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Abstract: Background: Acute promyelocytic leukaemia (APL) is a special subtype of acute myeloid leukaemia. The aim of this study was to investigate the anti-tumour effects of puerariae radix flavones (PRF), the total flavonoids from Chinese herb puerariae radix (PR), on the human APL cell line NB4 and to explore the potential mechanisms. Methods: NB4 cells were exposed to various concentrations (0, 10, 30, 50 μg/ml) of PRF. Cell viability was measured by MTT assay. The cell cycle, the extent of cell apoptosis and the mitochondrial transmembrane potential were analysed with flow cytometry (FCM). The expression of apoptosis-related proteins was assayed by western blot. Results: We found that PRF inhibited cell viability of NB4 cells in a time- and dose-dependent manner. The cell cycle was arrested in the G0/G1 phase. FCM assays showed that PRF induced apoptosis and decreased mitochondrial membrane potential in a dose-dependent manner. NB4 cells treated with PRF expressed higher levels of cleaved caspase-3, caspase-9 and poly ADP-ribose polymerase (PARP) with an increased Bax/Bcl-2 ratio and Bcl-xL expression. Western blot analysis showed that expression of JNK was significantly lower while the expression of p-JNK was greater. Further analysis showed activation of the JNK pathway of NB4 cells by PRF. Conclusions: Our data, taken together, indicate that PRF exerted a potent anti-tumour effect on NB4 cells by inducing apoptosis, the mechanism of which might be accompanied with the JNK pathway and the mitochondrial pathway activation.

Keywords: Puerariae radix flavones, NB4 cells, apoptosis, JNK pathway

Introduction

Acute promyelocytic leukaemia (APL) is a special type of acute myeloid leukaemia (AML) characterized by translocation of chromosomes 15 and 17, resulting in the fusion of the promyelocytic leukaemia (PML) gene to the retinoic acid receptor (RARα) gene [1, 20]. The currently used agents, all-trans retinoic acid (ATRA) and arsenic trioxide (ATO), directly target the PML-RARα oncoprotein and dramatically improve outcomes for APL patients, making APL highly curable [11, 25]. The research on the pathogenesis of APL and the targeted therapeutic mechanism can provide a theoretical basis and clinical reference for the study and treatment of leukaemia and other tumours. In the past decade, we also found that puerariae radix flavones (PRF), the main components of a Chinese herb, Puerariae radix (PR) could inhibit prolifer-ation of NB4 cells [17], a human APL cell line with t(15; 17) marker and maturation-inducible by ATRA.

Jun N-terminal kinases (JNKs), belonging to the super-family of mitogen-activated protein kinases (MAPKs), are involved in regulation of cell proliferation, differentiation and apoptosis [3]. JNK was reported to be activated in response to various extracellular stimuli, including growth factors, cytokines and cellular stressors such as heat shock, hyper-osmolarity and UV-radiation [12]. ATO is the most effective drug for treating APL in single doses. In *in vitro* experiments, JNK was activated during apoptosis of NB4 cells induced by ATO, while silencing JNK expression protected cancer cells from apoptosis induced by ATO *in vivo*. Therefore, JNK was thought to be an important signalling target in apoptosis of NB4 cells.

Our previous results suggested that the JNK signalling pathway could be the mechanism for NB4 cell apoptosis induced by PRF [27]. PRF can markedly inhibit the NB4 cells proliferation with arresting in S phase in vitro. Here, we explored the effects of PRF at lower concentrations (0-50 μg/ml) on the APL NB4 cell line and explored the possible molecular mechanisms.

Materials and methods

Drugs

PRF (80%) was purchased from Nanjing Ze Lang Biology Co., LTD (Nanjing, China) and kept as stock solution at 40 mg/mL in DMSO with the final concentration less than 0.5% during treatment. The stock solution was diluted in medium prior to use.

Cell culture

NB4 cells were obtained from Shanghai Institute of Hematology of RuiJin Hospital and cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Gibco) and incubated in a 37°C incubator with a humidified atmosphere containing 5% CO_2. Only cells in no mycoplasma contamination were used in the experiments.

MTT assay

A total of $5*10^4$ cells per well were seeded in 96-well plates and were treated with PRF at concentrations of 0, 10, 30, and 50 μg/ml for various time periods (24 h, 48 h or 72 h). After treatment, 0.1 mg of MTT was added to each well. The samples were incubated for 4 h, and the absorbance (optical density, OD value) was measured at 490 nm by spectrophotometry. Calculation of the cell growth inhibition rate at various concentrations was performed by comparing it with the growth rate of the control group. Inhibition rate = [1-OD value of treated cell/OD value of control cell] *100%.

Flow cytometric (FCM) analysis of apoptosis [26]

Apoptosis assessment was carried out by double staining the cell with FITC-annexin-V and propidium iodide (PI) as recommended by the manufacturer (BD Bioscience, USA). After 48-hour incubation with various concentrations of PRF, the cells were centrifuged twice in cold phosphate-buffered saline (PBS), resuspended in 1X binding buffer and incubated with 5 μl of FITC-conjugated annexin-V and 5 μl of PI for 15 min in the dark at room temperature. The cells were then analysed on a flow cytometer (Beckman Coulter, USA). Absorption wave length is 525 nm and emission wave length is 575 nm. Three independent experiments were performed.

Measurement of mitochondrial membrane potential (MMP) [19]

MMP was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (MultiSciences Biotech Co., Ltd.). Cells were treated with various concentrations of PRF for 48 h then washed with PBS and incubated in medium containing 2 μM JC-1 for 30 min at 37°C. The level of MMP was then analysed by flow cytometry excited by the 488 nm laser.

Cell cycle analysis

After 48 h of incubation with PRF, the cells were washed twice with PBS and incubated in 70% ice-cold ethanol before being stored at 4°C for more than 24 h. After being washed with PBS again, the cells were resuspended in PBS containing 50 μg/ml PI and 100 μg/ml RNase (BD Bioscience, USA). Fifteen minutes later, the cells were analysed by flow cytometry (Beckman Coulter). The proportion of cells in each stage of the cell cycle was determined using the Modfit software program. Three independent experiments were performed.

Western blot analysis

After 48-hour incubation with PRF, the cells were washed twice with cold PBS and lysed in cell lysis buffer for western blotting (Beyotime Institution of Biotechnology, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Beyotime Institution of Biotechnology). The protein concentration was determined using a BCA Protein Assay Kit (Beyotime, China). Forty to sixty micrograms of protein were separated by electrophoresis on 12% SDS-polyacrylamide gels (SDS-PAGE) and then electrotransferred onto polyvinyl difluoride (PVDF) membranes (Millipore Corporation, USA). The membranes were incubated for 2 h in TBST containing 5% non-fat dried milk and were then incubated with rabbit monoclonal antibody which included PARP (abcam, ab74290), caspase-3 (abcam, ab13585), caspase-9 (abcam,
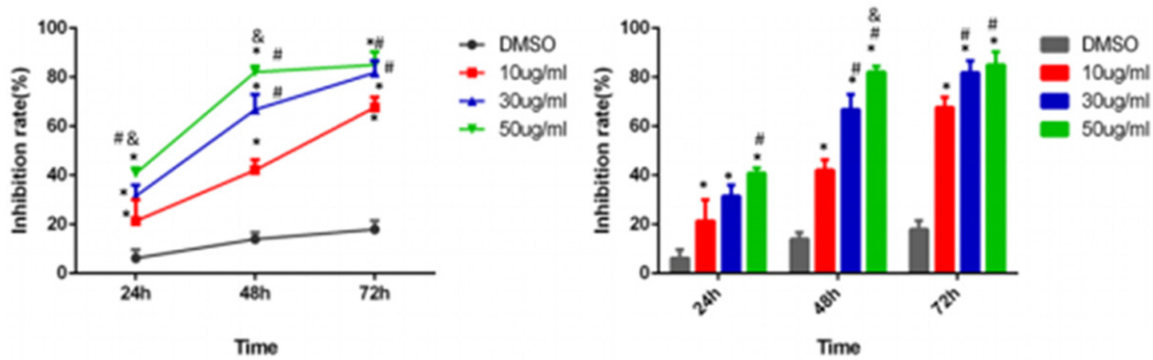
Figure 1. Treatment with PRF inhibited the proliferation of NB4 cells. MTT results demonstrated that PRF inhibited NB4 cell proliferation in a concentration- and time-dependent manner. $^{*}P<0.05$ vs control. $^{#}P<0.05$ vs 10 ug/ml, $^{&}P<0.05$ vs 50 ug/ml.
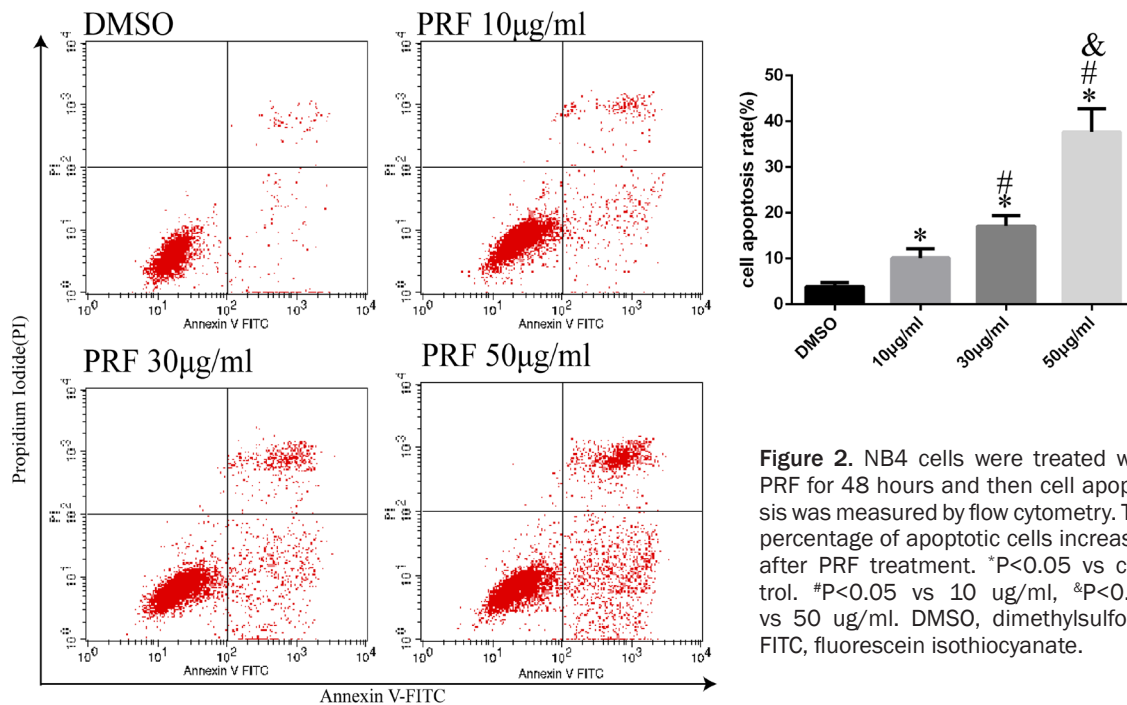

Figure 2. NB4 cells were treated with PRF for 48 hours and then cell apoptosis was measured by flow cytometry. The percentage of apoptotic cells increased after PRF treatment. $^{*}P<0.05$ vs control. $^{#}P<0.05$ vs 10 ug/ml, $^{&}P<0.05$ vs 50 ug/ml. DMSO, dimethylsulfode; FITC, fluorescein isothiocyanate.

ab32539), Bax (abcam, ab32503), Bcl-2 (abcam, ab32124), Bcl-xl (abcam, ab32370), ERK1/2 (abcam, ab184699), p-JNK (abcam, ab204085), p38 (abcam, ab31828), p-ERK (abcam, ab65142), p-p38 (abcam, ab4822), p-S-EK (abcam, ab132958), p-MKK4 (genetex, GTX50475) and c-Jun (genetex, GTX134395) (1:1000 dilution) at 4°C overnight. The membranes were washed three times with TBST and incubated with a goat anti-rabbit IgG secondary antibody (1:10000 dilution) for 2 h. The bands were detected using Chemiluminescent HRP Substrate (Millipore Corporation, USA) after the membranes were washed three times with TBST.

Statistical analysis

The data are presented as mean ± SD. SPSS 13.0 software (SPSS Inc., USA) was used to calculate a two-tailed Student's t-test and one-way ANOVA, which were used to compare the differences between the various treatments. A *P* value of less than 0.05 was considered statistically significant.

Results

PRF inhibited proliferation of NB4 cells

Exposure of NB4 cells to escalating concentrations of PRF (0, 10, 30, and 50 µg/ml) for 24,
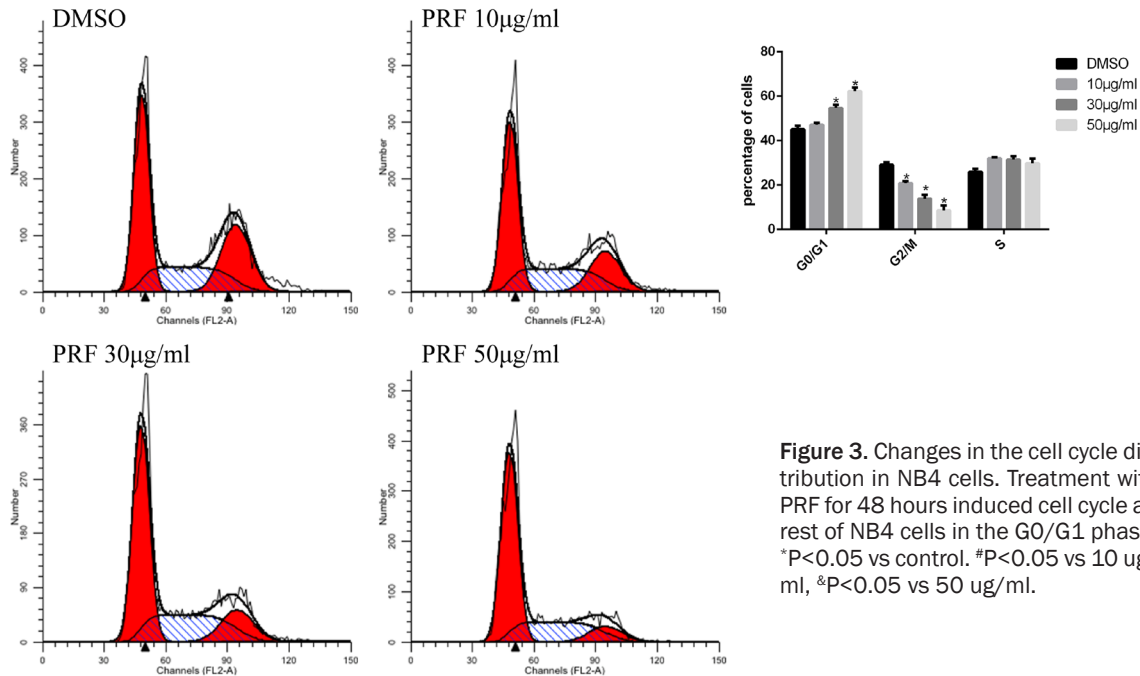
Figure 3. Changes in the cell cycle distribution in NB4 cells. Treatment with PRF for 48 hours induced cell cycle arrest of NB4 cells in the G0/G1 phase. $^*P<0.05$ vs control. $^\#P<0.05$ vs 10 ug/ml, $^\&P<0.05$ vs 50 ug/ml.
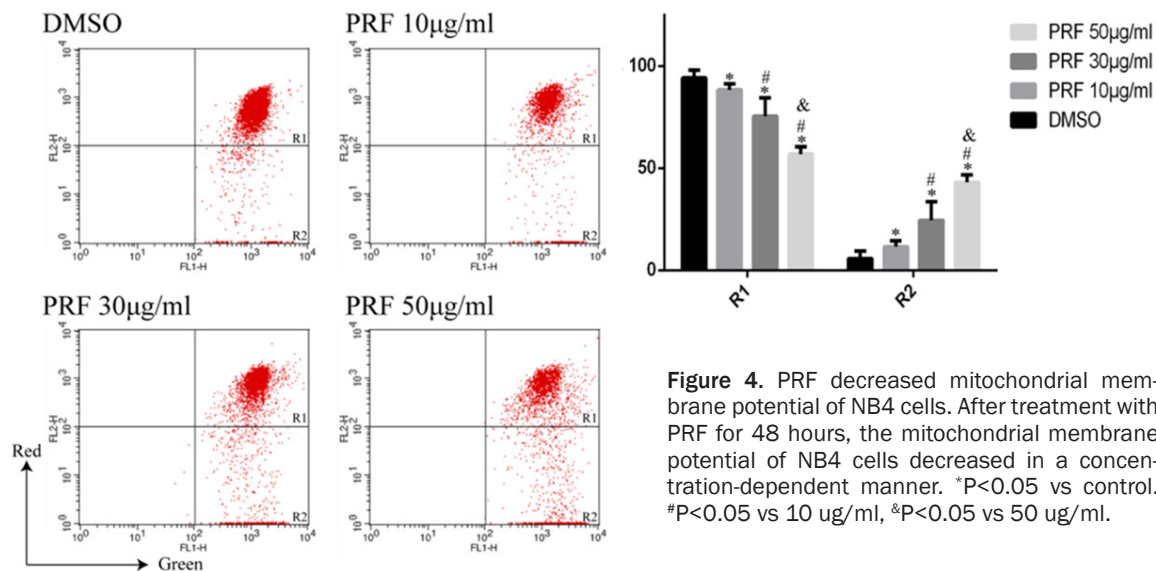

Figure 4. PRF decreased mitochondrial membrane potential of NB4 cells. After treatment with PRF for 48 hours, the mitochondrial membrane potential of NB4 cells decreased in a concentration-dependent manner. $^*P<0.05$ vs control. $^\#P<0.05$ vs 10 ug/ml, $^\&P<0.05$ vs 50 ug/ml.

Table 1. The cell apoptosis percentage of the flow cytometry results of NB4 cells treated with PRF for 48 hours

	DMSO	PRF 10 µg/ml	PRF 30 µg/ml	PRF 50 µg/ml
Early apoptosis	4.90%	5.45%	8.47%	16.77%
Late apoptosis	1.91%	3.82%	6.27%	15.14%

48 or 72 h, as shown in **Figure 1**, inhibited cell growth in a concentration- and time-dependent manner, as measured by MTT assay.

PRF induced apoptosis in NB4 cells

FCM assays were carried out to test whether PRF induced apoptosis in NB4 cells. NB4 cells previously treated with PRF (0, 10, 30, and 50 µg/ml) for 48 h were collected and stained with annexin-V and PI. As shown in **Figure 2**, the rate of apoptosis, especially early apoptosis, was significantly higher in the treated group than in the control group. Moreover, PRF induced apoptosis in NB4 cells in a concentration-dependent manner.
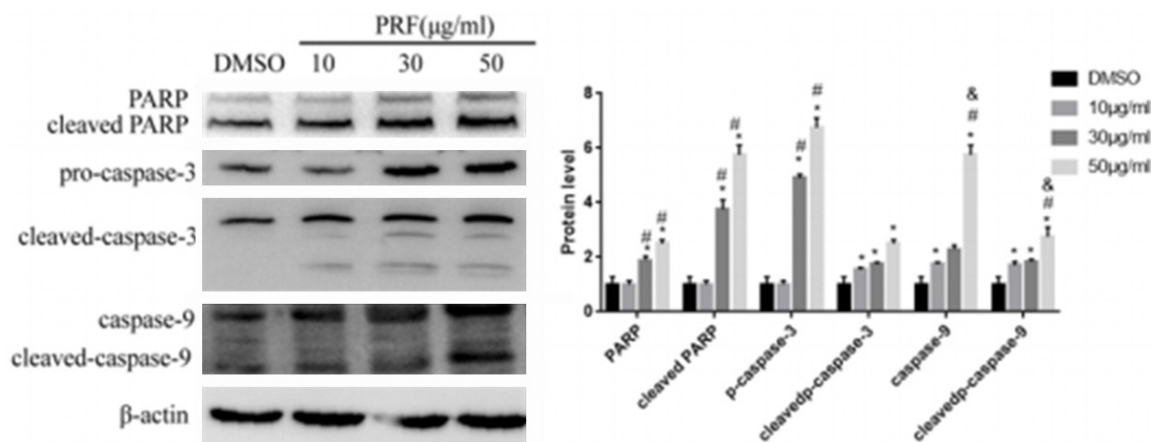
Figure 5. Effect of PRF on the expression of apoptosis-related proteins. NB4 cells were treated with PRF for 48 hours. The expression of cleaved PARP and cleaved-caspase-3 increased while caspase-3 decreased. *$P < 0.05$ vs control. #$P < 0.05$ vs 10 ug/ml, &$P < 0.05$ vs 50 ug/ml.
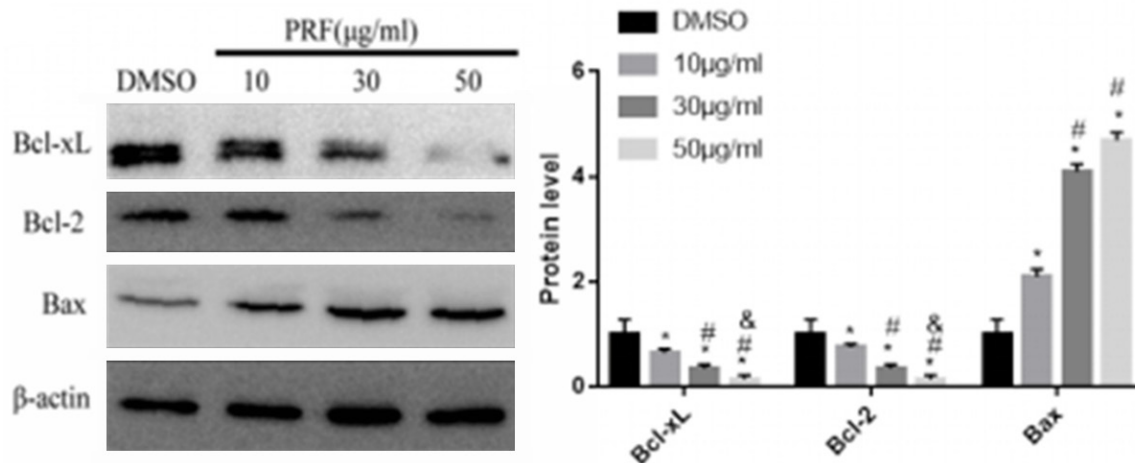

Figure 6. Effect of PRF on the expression of apoptosis-related proteins. NB4 cells were treated with PRF for 48 hours. The expression of Bax increased whereas Bcl-xL and Bcl-2 decreased. *$P < 0.05$ vs control. #$P < 0.05$ vs 10 ug/ml, &$P < 0.05$ vs 50 ug/ml.

PRF induced G0/G1 phase cell cycle arrest in NB4 cells

To examine whether PRF induced redistribution of the cell cycle of NB4 cells, the effects of various concentrations of PRF on cell cycle after 48 hours of intervention were measured. The results are shown in **Figure 3**. PRF induced G0/G1 phase arrest and the percentage of NB4 cells in the G0/G1 phase significantly increased in response to PRF (30 and 50 µg/ml) treatment for 48 h.

PRF decreased MMP in NB4 cells

The effects of PRF on the MMP in NB4 cells were investigated after 48 hours of interven-

tion. We used JC-1 fluorescent dye and flow cytometry to measure the effects of various concentrations of PRF. As shown in **Figure 4** and **Table 1**, increasing doses of PRF for 48 h led to a significant reduction in MMP, suggesting that PRF-induced apoptosis was mitochondria-dependent.

PRF increased the expression levels of apoptosis regulatory proteins in NB4 cells

Western blotting was used to measure the effects of PRF on apoptosis-related proteins PARP, caspase-3, caspase-9, Bax, Bcl-2, Bcl-xl. PRF induced a dose-dependent cleavage of PARP, caspase-3 and caspase-9 after 48 hours of intervention (**Figure 5**). In addition, the
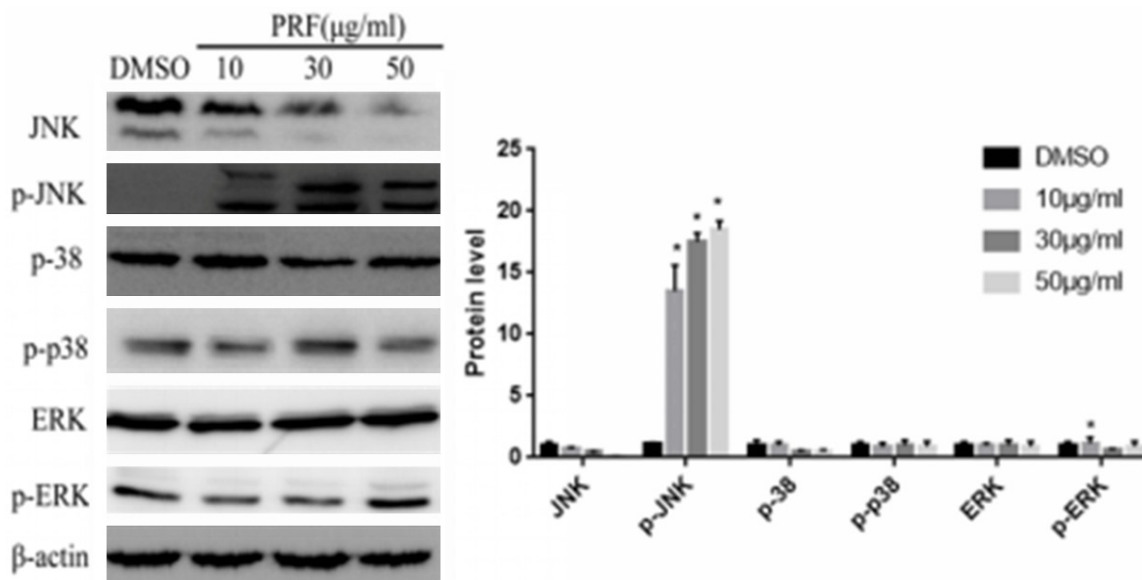
Figure 7. Effect of PRF on the expression of mitogen-activated protein kinases proteins. NB4 cells were treated with PRF for 48 hours. The expression of p-JNK increased while JNK was decreased. The expression of p-ERK increased with only 10 μg/ml PRF, and p-p38 was not affected by PRF. $^*P<0.05$ vs control. $^\#P<0.05$ vs 10 ug/ml, $^\&P<0.05$ vs 50 ug/ml.
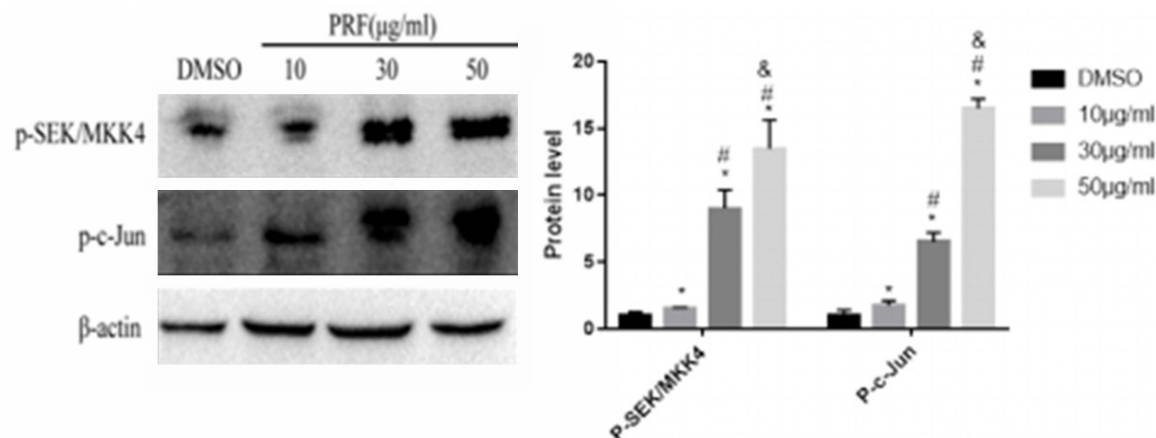

Figure 8. Effect of PRF on the expression of JNK pathway proteins. NB4 cells were treated with PRF for 48 hours. The expression of p-SEK/MKK4 and p-c-Jun increased. $^*P<0.05$ vs control. $^\#P<0.05$ vs 10 ug/ml, $^\&P<0.05$ vs 50 ug/ml.

expression of proapoptotic protein Bax increased with the dose, while expression of the apoptosis-inhibitory proteins Bcl-2 and Bcl-xl decreased (**Figure 6**). These results suggest that PRF did not induce apoptosis of NB4 cells from the protein level.

PRF regulated the JNK pathway in NB4 cells

MAPKs are important for regulating cell apoptosis. ERK, JNK, p38 MAPK are family members of MAPKs. To investigate the intrinsic mechanism of PRF-induced apoptosis of NB4 cells, we evaluated the effect of PRF on MAPK expres-

sion after 48 hours of intervention. PRF significantly increased phosphorylation of JNK, where-as the expression of p-ERK increased only after treatment with 10 μg/ml PRF, and p-p38 was not affected by PRF (**Figure 7**). Therefore, we further examined the expression of the JNK upstream protein SEK/MKK4 and downstream protein c-Jun. We found that phosphorylation of SEK/MKK4 and c-Jun was greater after 48 hours of PRF intervention (**Figure 8**), indicating that the mechanism of apoptosis induced by PRF in NB4 cells may be related to activation of the JNK pathway.

Discussion

PR, the dried root of Pueraria lobate (Willd.) Ohwi, is widely used to treat alcoholism, hypertension, common colds and influenza, and it is also used to improve vascular activity [13]. Studies have shown that PRF, the main active ingredient of PR, has potential anti-tumour activity. It inhibited proliferation of a variety of tumour cells such as human breast cancer and induced their apoptosis [9, 18, 23]. In the present study, we found that 0-50 µg/ml PRF inhibited proliferation of NB4 cells in a concentration- and time-dependent manner. FCM and TUNEL analysis also confirmed apoptosis of NB4 cells and cell cycle arrest in the G0/G1 phase induced by 0-50 µg/ml PRF.

Almost all tumours have characteristics of excessive proliferation, differentiation failure and apoptosis disorders resulting from destruction of the mechanisms for cell cycle regulation. In the present study, FCM analysis of cell cycle showed that after inhibition by PRF, NB4 cells in the G0/G1 phase increased, indicating that PRF affected cell cycle regulation and arrested cells in the G0/G1 phase.

Western blot analysis further showed that the expression of cleaved-PARP and cleaved-caspase-3 were significantly elevated. Chemotherapy drugs exert anti-tumour effects mainly by inducing apoptosis of tumour cells. Apoptosis is a precisely controlled multi-step event that results in cell death [4, 21]. There are two main pathways that initiate apoptosis: the extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The extrinsic pathway can be initiated in conjunction with death receptors on the cell membrane surface, including Fas/CD95, tumour necrosis factor receptor (TNFR) and its corresponding ligands FasL and TNFα, forming death-inducing signal complexes (DISC). DISC is responsible for recruitment and activation of caspase-8. The intrinsic pathway is characterized by attenuation of the MMP, increasing membrane permeability and release cytochrome c into the cytoplasm. Cytochrome c can form the apoptosome with caspase-9 precursors that in turn activate caspase-9. The activation of caspase-8 and caspase-9 both perform apoptosis by activating the apoptotic executive protein caspase-3 [2, 16]. Hence, caspase-3 is considered as the most important of the execu-

tioner caspases and a marker protein of apoptosis. Its activation cleaves various substrates including PARP, ultimately causing the morphological and biochemical changes seen in apoptotic cells. Therefore, our result confirmed the pro-apoptosis effect of PRF from the protein level in terms of cleaved-PARP and cleaved-caspase-3.

The FCM result showed that PRF decreased the MMP of NB4 cells in a concentration-dependent manner. Western blot showed increased expression of caspase-9 after PRF intervention. Mitochondria are the control centres of cell life activities, the cellular respiration chain and oxidative phosphorylation, as well as the regulatory centre of apoptosis [15]. The decrease in MMP is considered to be the earliest event in the process of apoptotic cascade. Once the MMP changes dramatically, apoptosis is irreversible. Therefore, apoptosis induced by PRF may be related to the activation of intrinsic apoptotic pathways.

The Bcl-2 protein expression was lower and the Bax protein expression was higher after 10-50 µg/ml PRF intervention, suggesting that PRF-induced apoptosis of NB4 may activate the intrinsic pathway by regulating the Bax/Bcl-2 ratio. Disruption of the function of Bcl-2 protein led to permeabilization of the mitochondrial membrane [10]. Both pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 play important roles in the intrinsic pathway.

We further measured phosphorylation levels of JNK, p38 and ERK (p-JNK, p-p38, p-ERK and p-SEK/MKK4). After 10-50 µg/ml PRF treatment, the expression of JNK was lower while the expression of p-JNK, p-SEK/MKK4 were higher. However, the expression of p-38, ERK, p-p38 and p-ERK was unchanged. MAPKs compose a family of protein kinases whose function and regulation are highly conserved. MAPKs control many important cellular functions by phosphorylating specific serines and threonines of target protein substrates. JNK, p38 and ERK are three main signalling pathways in the MAPKs family. JNK, known as the stress-activated protein kinase (SAPK) of the MAPK family, is initially activated in response to a variety of stress signals and has been implicated in several cellular events, including apoptosis. JNK activation is mediated by the upstream

MKK4 and MKK7 kinase via dual phosphorylation of the Thr-Pro-Tyr motif [6]. Upon activation, MKKs phosphorylate and activate SAPK/JNK kinase, which then translocates to the nucleus and regulates transcription via acting on c-Jun, ATF-2 and other transcription factors [5]. The role of the JNK pathway in apoptosis has been extensively studied [8, 28]. A report indicated that JNK activation is a critical step for the chemotherapy drug to trigger apoptosis in AML [22]. Hence, the JNK signalling pathway could be the molecular mechanism of the apoptotic effect of 10-50 µg/ml PRF on the NB4 cell line.

In conclusion, our study evaluated the anti-proliferative effect of PRF in NB4 cells, which was mediated by the induction of apoptosis, possibly via the JNK signalling pathway. Our findings might significantly contribute to the understanding of the anti-cancer properties of PRF as well as future development of such compounds as potential therapeutic agents for treating APL and possibly relapsed APL.

Acknowledgements

Disclosure of conflict of interest

None.

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