RESEARCH ARTICLE

Effect of Withaferin A on A549 Cellular Proliferation and Apoptosis in Non-small Cell Lung Cancer

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Abstract

Objective: To explore the effect of Withaferin A on A549 cellular proliferation and apoptosis in non-small cell lung cancer (NSCLC). Materials and Methods: NSCLC cell line A549 was selected to explore the effect of Withaferin A on A549 cellular proliferation, apoptosis and the PI3K/Akt signal pathway capable of regulating tumor biological behavior by assessment of cellular proliferation, cellular apoptotic rates and cellular cycling as well as by immuno-blotting. Results: Withaferin A could inhibit A549 cellular proliferation and the control rate was dosage-dependent ($P<0.05$), which also increased time-dependently with the same dosage of Withaferin A ($P<0.05$). The apoptotic indexes in A549 cells treated with 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A for 48 h were significantly different ($P<0.05$). In addition, the apoptotic rates of each group in both early and advanced stages were higher than those in 0 μmol·L^{-1} ($P<0.05$), which were evidently higher after 48 h than those after 24 h ($P<0.05$). A549 cells treated by Withaferin A for 48 h were markedly lower in Bcl-2 level and obviously higher in Bax and cleaved caspase-3 levels than those treated by 0 μmol·L^{-1} Withaferin A ($P<0.05$), and there were significant differences among 5, 10 and 20 μmol·L^{-1} Withaferin A ($P<0.05$). The ratios of A549 cells treated by Withaferin A for 48 h in G_0/G_1 stage were higher than those in 0 μmol·L^{-1} , while those in S and G_2/M stages were obviously lower than those in G_2/M stage, and there were significant differences in 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A ($P<0.05$). Additionally, p-Akt/Akt values were in reverse association with dosage, and the differences were significant ($P<0.05$). Conclusion: Withaferin A can inhibit the proliferation and apoptosis of A549 cells by suppressing activation of the PI3K/Akt pathways.

Keywords: Non-small cell lung cancer - proliferation - apoptosis - A549 cells

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Introduction

Lung cancer is the leading cause for tumor-associated deaths, in which non-small cell lung cancer (NSCLC) accounts for 85% and most patients are in advanced stages with poor therapeutic efficacy when diagnosed (Jemal et al., 2010; Cho et al.,2013; Lu et al., 2013; Wang et al., 2013; Yan et al., 2013; Kim et al., 2014). Though there has been great development in clinical treatment of NSCLC, its prognosis is still unsatisfactory due to the low rate of 5-year survival in clinic (Verdecchia et al., 2007). Therefore, seeking more effective drugs for NSCLC is of great significance in its clinical treatment. Withaferin A is an active ingredient extracted from Withania omnifera (Yu et al., 2013), which possesses the functions of regulating immunity and anti-inflammation, etc.. In recent years, it has also been discovered that Withaferin A has anti-tumor activity and is effective in inhibiting multiple tumors, such as breast cancer, etc. (Hahm et al., 2013; Lee et al., 2013). NSCLC cell line A549 was selected in this study to explore the effect of Withaferin A on A549 cellular proliferation, apoptosis and PI3K/Akt signal pathways capable of regulating tumor biological behaviors, and the results are as follows.

Materials and Methods

Agents

A549 cell lines were obtained from American ATCC; Withaferin A, dimethyl sulfoxide (DMSO), methyl thiazolyl tetrazolium (MTT), Sodium dodecyl sulfate (SDS) and Tris were purchased from American Sigma-Aldrich company; RPMI 1640 culture medium, Fetal calf Serum (FCS) and trypsase were brought from American Gibco company; penicillin and Streptomycin were gained from Hyclone company; glycine, RNase, Acrylamide and methylene-bisacrylamide were received from American Amresco company; Annexin-FITC/PI kits for apoptotic detection was brought from Keygen Biotech. Co., Ltd; internal references GAPDH, Bcl-2 and Cleaved caspase-3 polyclonal antibodies were obtained from American Cell Sigaling Technology company; Bax, Akt and *P*-Akt

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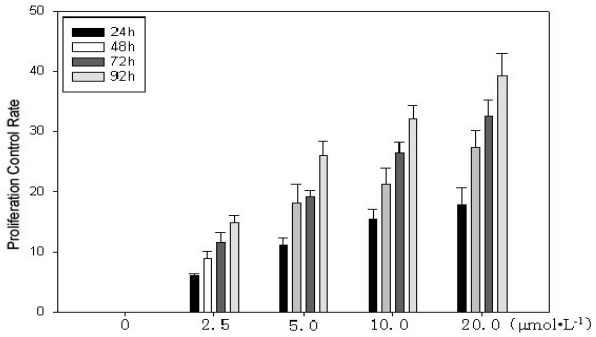

Figure 1. Control Rate of Cellular Proliferation Treated with Different Dosages of Withaferin A

polyclonal antibodies were purchased from Santa Cruz company; and Hoechst 33258 was brought from Beyotime Institute of Biotechnology.

Cellular proliferation detection

A549 cells in logarithmic phase were treated by 0.25% trypsase, made into mono-cellular suspension by blowing and beating, inoculated in 96-well plate by 1×10^5/well for totally 200 μL and cultured for 24 h, added with 20 μL 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A and cultured for 24, 48, 72 and 96 h respectively in incubators containing 5% CO_2 saturated humidity at 37°C, added with 10 μL 5 mg·mL^{-1} MTT solution in each well, and cultured for 4 h. Optical density A492 in each well under 492 nm was measured by ELIASA and it was set up as 100% in control group. Three parallel holes were established in each group and the study was repeated for 3 times to obtain the mean values. Control rate (%)=[(control group-blank group)- (medicine group-blank group)]/ (control group-blank group)×100%.

Apoptotic index detection

A549 mono-cellular suspension was inoculated on sterilized slide placed in the bottom of 12-well culture plate, and culture medium was discarded after cellular adherence presented. Then culture mediums containing different dosages of Withaferin A were added and cultured for 48 h, added with paraformaldehyde to fix cells, washed by PBS, added with 5 μg·mL^{-1} Hoechst 33258 and stained for 30 min at room temperature shielded from light. Fluorescent microscope was used to observe cellular morphology and 5 high-power fields were selected to calculate the counts of apoptotic cells and total cells, so as to obtain the apoptotic indexes. Apoptotic index=Apoptotic cell count/Total cell count×100%.

Detections of cellular apoptotic rate and cellular cycle

A549 cells were inoculated in 6-well culture plate by 1×10^6/well, added with 20 μL 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A and cultured for 24 h in 5% CO_2 incubator at 37°C respectively, washed by PBS once based on the instruments of apoptosis detection kit, added with Annexin V/FITC and PI, and stained for 15 min to calculate apoptotic rate and cellular cycle by flow cytometry.

Immuno-blotting method

A549 cells treated by different dosages of Withaferin A for 48 h were collected and centrifuged at high-speed, supernatant was discarded and cells were decomposed on ice to determine the protein levels by BAC method. The protein samples were mixed with loading buffer and given 10% SDS-PAGE gel electrophoresis under routine methods. The constant voltages of spacer and separation gels were 80 V (for 30 min) and 100 V (about 80 min), respectively. Then, the samples were transformed to PVDF membrane in semi-dry condition, added with appropriate amounts of p-Akt primary antibodies (1: 200), Bax, Caspase-3 and Bcl-2 (1: 300), incubated at 4°C overnight, added with secondary antibodies (1: 3000) and incubated for 2 h in room temperature by shaking, which were colored by ECL and developed to analyze the optical intensity of each developed stripe by Gel-Pro analyzer. p-Akt was the ratio of solved solution with Akt while the relevant expressions of other proteins were the ratios with GAPDH.

Table 1. Effect of Withaferin A on Cellular Apoptosis (%, $\bar{\chi}\pm s$)

Groups	24 h		48 h	
	Early stage	Advanced stage	Early stage	Advanced stage
0 μmol·L^{-1}	3.18±0.53	1.16±0.73	4.42±1.26	2.21±0.93
2.5 μmol·L^{-1}	6.72±1.90^a	3.25±1.19^a	11.63±2.48^{ab}	6.38±1.42^{ab}
5.0 μmol·L^{-1}	11.91±2.91^a	6.84±1.35^a	13.57±2.35^a	9.27±1.55^{ab}
10.0 μmol·L^{-1}	17.08±4.18^a	8.29±2.51^a	22.29±4.70^{ab}	10.65±2.82^a
20.0 μmol·L^{-1}	21.37±3.37^a	11.68±2.94^a	28.68±5.58^{ab}	15.63±2.73^{ab}

Compared with 0 μmol·L^{-1}, $^aP<0.05$; Compared with 24 h, $^bP<0.05$

Results

Effect of Withaferin A on cellular proliferation

A549 cells were treated by 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A for 24 h, 48 h, 72 h and 96 h, respectively, and the MTT laboratory results showed that Withaferin A could inhibit the proliferation of A549 cells, which was in positive association with the dosages and in dosage-dependent relationship ($P<0.05$). In addition, in the same dosage of Withaferin A, the cellular control rate also increased time-dependently ($P<0.05$) (Figure 1).

Effect of Withaferin A on cellular apoptosis

Cellular apoptotic indexes: The apoptotic indexes of A549 cells treated respectively by 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A for 48 h were (2.75±0.64)%, (4.61±1.36)%, (9.75±2.78)%, (12.92±3.42)% and (18.68±4.31)% respectively, and the differences were all significant ($P<0.05$) (Figure 2).

Cellular apoptotic rate: A549 cells were treated by 0, 2.5, 5.0, 10.0 and 20.0μmol·L^{-1} Withaferin A for 24 h and 48 h respectively, and the Annexin-FITC/PI double-staining method indicated that the apoptotic rates in early and advanced stages of each dosage were all evidently

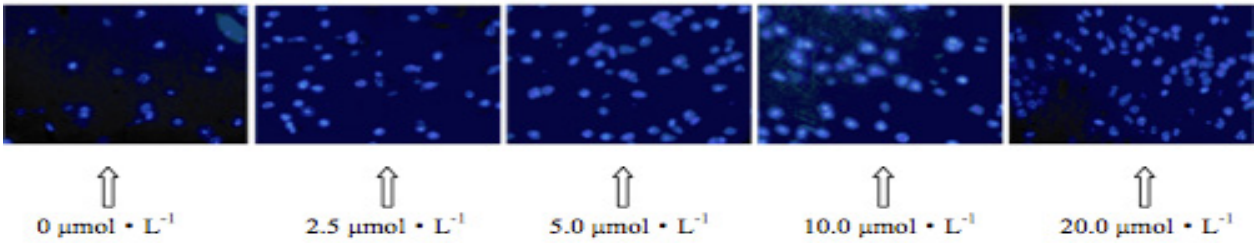

Figure 2. Primary Figures of Apoptosis after Treatment in each Group

Table 2. Effect of Withaferin A on Apoptosis-Associated Gene Expression (%, $\bar{\chi}$±s)

Groups	Bcl-2	Bax	Cleaved caspase-3
0 μmol·L^{-1}	0.72±0.23	0.32±0.09	0.28±0.14
2.5 μmol·L^{-1}	0.68±0.19	0.36±0.18	0.35±0.19
5.0 μmol·L^{-1}	0.46±0.17^a	0.43±0.20^a	0.47±0.22^a
10.0 μmol·L^{-1}	0.33±0.13^a	0.52±0.21^a	0.54±0.27^a
20.0 μmol·L^{-1}	0.27±0.09^a	0.60±0.24^a	0.67±0.20^a

Compared with 0 μmol·L^{-1}, ^aP<0.05

Table 3. Cellular Cycle Distribution after 48 h Treatment with Withaferin A (%, $\bar{\chi}$±s)

Groups	G_0/G_1	S	G_2/M
0 μmol·L^{-1}	42.76±4.28	39.87±3.29	16.40±2.38
2.5 μmol·L^{-1}	47.76±2.59	33.52±3.52	12.40±2.25
5.0 μmol·L^{-1}	52.90±3.32^a	29.16±1.79^a	8.15±1.26^a
10.0 μmol·L^{-1}	61.17±4.79^a	27.58±1.84^a	7.28±2.68^a
20.0 μmol·L^{-1}	68.93±5.30^a	24.10±1.29^a	5.46±1.13^a

Compared with 0 μmol•L^{-1}, ^aP<0.05

higher than in 0 μmol·L^{-1} Withaferin A (P<0.05). And except the early apoptotic rate in 5.0 μmol·L^{-1} and advanced one in 10.0 μmol·L^{-1} Withaferin A, the apoptotic rates of A549 cells treated by 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A for 48 h were all markedly higher than those for 24 h, and there were significant differences (P<0.05) (Table 1).

Influence of apoptosis-associated gene expression: A549 cells treated by each dosage of Withaferin A (except 2.5 μmol·L^{-1}) for 48 h were lower in Bcl-2 expression and higher in Bax and Cleaved caspase-3 expressions than those in 0 μmol·L^{-1}, respectively, and the differences among 5, 10 and 20 μmol·L^{-1} were significant (P<0.05) (Table 2).

Effect of Withaferin A on cellular cycle

The ratios of A549 cells treated by different dosages of Withaferin A for 48 h in G_0/G_1 stage were higher than those in 0 μmol·L^{-1}, while those in S and G_2/M stages were obviously lower than in G_2/M stage, and there were significant differences in cellular cycle distribution treated by 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A (P<0.05) (Table 3).

Effect of Withaferin A on PI3K/Akt signal pathway

The p-Akt/Akt values of A549 cells treated by 0, 2.5, 5.0, 10.0 and 20.0 μmol·L^{-1} Withaferin A for 48 h were (0.32±0.17), (0.26±0.12), (0.18±0.09), (0.12±0.04) and (0.06±0.01), respectively, which were reversely proportional to dosages, and the differences were significant (P<0.05).

Discussion

Withaferin A is a natural anti-tumor agent, however, its mechanism of inhibiting the cellular proliferation of multiple tumors is still unclear, which may be correlated with inducing cellular apoptosis, inhibiting DNA synthesis and impacting cellular signal pathways (Hahm et al., 2013; Lee et al., 2013). Eun-Ryeong et al (Hahm et al., 2013) proposed that mitogen activited protein kinase (MAPK) and Mcl-1 played an important part in the process of inducing cellular apoptosis of breast cancer by Withaferin A, which was proved by the activation of MAPK pathways in cellular experiments. Li et al. (2013) found that Withaferin A could induce the apoptosis of GBC-SD cells in human gallbladder carcinoma by impacting cellular cycles, such as changing the expressions of periodic elements, etc.. And Roy et al. (2013) discovered that Withaferin A could influence the cellular proliferation of prostate gland so as to inhibit its growth. All studies above ensured that Withaferin A can inhibit cellular activity of multiple tumors through various ways.

In this study, it was found that Withaferin A could evidently inhibit A549 cellular proliferation time- and dosage-dependently in that the inhibiting rate of A549 cellular proliferation increased along with the increase of dosages and time, which was consistent with the researches of Withaferin A on other tumors (Hahm et al., 2013; Roy et al., 2013). Moreover, this study also verified the effect of Withaferin A on A549 cellular apoptosis, including apoptotic indexes, apoptotic rates and apoptosis-associated gene expressions by many methods, which demonstrated that Withaferin A could induce the apoptosis of A549 cells, and had obvious influence on cellular apoptosis in all dosages except 2.5 μmol·L^{-1}, and the differences were significant. Bax and cleaved-Caspase-3 are apoptosis-promoting genes, in which Bcl-2 is the most common one in inhibiting apoptosis (Liu et al., 2010; Kim et al., 2012), while the apoptotic condition is often evaluated by the changes of above 3 protein levels. Additionally, this study suggested that Withaferin A could elevate Bax and cleaved-Caspase-3 levels and reduce Bcl-2 level, improving the cellular apoptosis on molecular level.

PI3K/Akt signal pathways are critical in cellular proliferation and apoptosis, which are activated in most tumor cells (Osaki et al., 2004), leading to increased p-Akt, a formation in phosphorylated Akt induced by PI3K. However, the ratio of p-Akt may decrease if PI3K/Akt signal pathways are not activated. In this study, p-Akt/Akt decreased after 48 h treatment with Withaferin A,

indicating that Withaferin A could inhibit the activity of PI3K/Akt signal pathways, therefore, it was predicated that Withaferin A might inhibit the proliferation and apoptosis of A549 cells by suppressing PI3K/Akt signal pathways.

In conclusion, Withaferin A can inhibit the proliferation and apoptosis of A549 cells by suppressing PI3K/Akt signal pathways, which has certain prospect in treating NSCLC.

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