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Original Research Article

Angelica sinensis polysaccharide promotes apoptosis by inhibiting JAK/STAT pathway in breast cancer cells

Zhaoyuan Fu[1], Yingdong Li[1]*, Suisheng Yang[2], Chengxu Ma[1], Rongrong Zhao[3], Huan Guo[1], Huiping Wei[1]

[1]Department of Integrated Traditional Chinese and Western Medicine, Gansu University of Traditional Chinese Medicine, Lanzhou City 730000, [2]Department of Breast Oncology, Gansu Cancer Hospital, Lanzhou City 73005, [3]Department of Clinical Laboratory Medicine, Affiliated Hospital of Gansu University of Traditional Chinese Medicine, Lanzhou City, Gansu Province 730000, China

For correspondence: **Email: YingdongLigtf@163.com; **Tel:** +86-931-8635100*

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Abstract

Purpose: *To determine whether Angelica polysaccharide (APS) induced apoptosis via regulation of the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in breast cancer cells.*
Methods: *Human MCF-7 cells were treated with APS. Cell proliferation, apoptosis, expression of apoptotic proteins, and the phosphorylation level of Janus kinase (JAK) and STAT were measured, respectively. For further analysis, MCF-7 cells were transfected with a JAK2 overexpression plasmid or treated with a classical JAK inhibitor, ruxolitinib.*
Results: *Treatment with APS dose-dependently reduced cell proliferation, induced apoptosis, and downregulated the levels of phosphorylated JAK and STAT in MCF-7 cells. JAK inhibitor, ruxolitinib, blocked JAK/STAT pathway and induced cell apoptosis in MCF-7 cells. Besides, JAK2 overexpression reversed the effects of APS on cell viability and apoptosis.*
Conclusion: *The results indicate that polysaccharide isolated from Angelica sinensis promotes apoptosis by inhibiting JAK/STAT pathway in breast cancer cells. Thus, APS may be useful as a potential therapeutic agent for breast cancer.*

Keywords: *Angelica polysaccharide, Apoptosis, Breast cancer, JAK, STAT*

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INTRODUCTION

Breast cancer, the most prevalent malignancy, is the second leading cause of cancer death in women [1]. Despite the significant achievement in recent decades, breast cancer is estimated to account for 30% of new cancer diagnoses in women [2]. Apoptosis has an important role in the carcinogenesis and treatment of breast cancer [3], because malignant cells usually lose the ability to undergo programmed cell death, which resulting in enhanced cell proliferation, tumor growth, and therapeutic resistance [4]. Research on breast cancer has focused on the regulation of intracellular signaling pathways that induce apoptosis and overcome cell death

resistance.

It has been reported that deregulation of the signal transducers and activators of transcription (STAT) signaling pathway leads to imbalanced proliferation, apoptosis and differentiation in various solid tumors [5]. STAT3 is one of the seven members in the STAT family. Behara *et al* showed that, in human breast cancer cells, the phosphorylation of Janus kinase 2 (JAK2) and STAT3 was activated to regulate apoptosis and tumor growth [6]. Inhibiting JAK2/STAT3 pathway could reduce the growth of breast cancer cells and xenografts, suggesting this pathway could be a promising therapeutic target for breast cancer [7].

Angelica sinensis is a traditional Chinese herbal medicine with anti-tumor and anti-proliferative effects in various cancer cell types [8,9]. Angelica polysaccharide (APS), the main bioactive compound in *Angelica sinensis*, has the ability to inhibit tumor growth both *in vitro* and *in vivo* [10]. The signaling pathways that involved in APS-induced apoptosis in cancer cells have been explored. For example, *Cao et al* found that APS stimulated apoptosis and inhibited proliferation in HeLa cells by regulating the expression of B-cell lymphoma 2 (*BCL-2)* family members, such as BCL-2 and BCL-2-associated X protein *(BAX) [11].* Zhou *et al* showed that APS induced T47D cancer cell apoptosis via the cyclic AMP response element-binding protein (CREB) signaling pathway [12]. Importantly, a study on iron deficiency in anemic rats showed that APS prevented the activation of the JAK signaling pathway [13]. Based on these investigations, this study aimed to investigate whether APS induced breast cancer cells apoptosis by inhibiting the JAK2/STAT3 pathway.

EXPERIMENTAL

Extraction of APS and cell line

The roots of *Angelica sinensis* (Oliv.) Diels were from Minxian County, Gandu Province, China and the APS was extracted from *A. sinensis* as previously described [14]. The percentage of total sugar in the extracted APS was 91.5 %, as determined by the phenol-sulfuric acid method [15], and the component sugars were glucose and arabinose at a molar ratio of 13.8:1. MCF-7 (human adenocarcinoma breast cancer cell line,#HTB-22, ATCC, Manassas, USA) was cultured in RPMI 1640 medium supplemented with fetal bovine serum (10 %) and penicillin (1 %) and streptomycin (Sigma, St. Louis, USA) in a 5 % CO_2 humidified atmosphere(37 °C).

MTT assay

The MCF-7 cells were seeded into 96-well plates (5×10^3 cells per well) (Thermo Fisher Scientific, Waltham, USA) and incubated overnight. Next, cells were treated with 10, 30, 50, or 100 μg/mL APS or with 20 μM ruxolitinib. After incubated for 1, 2, 3, or 4 days, MTT (10 μL, 0.5 mg/mL, Sigma, St. Louis, USA) was added and incubated for 4 h. Absorbance of the wells was measured using a microplate reader (at 490 nm, Bio-rad, Hercules, USA).

Cell apoptosis assay

The MCF-7 cells were placed into 6-well plates (2×10^5 cells per well) (Thermo Fisher Scientific, Waltham, USA) and incubated with 10, 30, 50, or 100 μg/mL APS or with 20 μM ruxolitinib for 96 h. The cells were centrifugated and resuspended in binding buffer (500 μL). Then, V-FITC (5 μL) and propidium iodide (5 μL, Biovision Research Products, Mountain View, USA) were added to the cell suspensions and incubated (5 min, 37 °C, dark). A Navios flow cytometer was used for assay and the results were analyzed by Kaluza software version 1.3 (Beckman Coulter, Brea, USA).

Western blot analysis

MCF-7 cells were seeded into 6-well plates (2×10^5 cells per well) and incubated with 10, 30, 50, or 100 μg/mL APS or with 0 or 20 μM ruxolitinib for 96 h. Cells were then lysed in RIPA buffer with protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, USA). The protein concentrations were measured and then normalized for protein content (Pierce, Rockford, USA). The cell lysates were separated using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and then transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were incubated with the following antibodies at a 1:1,000 dilution: BCL-2 (#ab59348, Abcam, Cambridge, UK), cleaved RARP (#5625, Cell Signaling), BAX (#ab32503, Abcam), cleaved caspase-3 (#9664, Cell Signaling, Danvers, USA), p-JAK2 (#3771, Cell Signaling), JAK2 (#3230, Cell Signaling), p-STAT3 (#9145, Cell Signaling), STAT3 (#9139, Cell Signaling), myeloid cell leukemia (MCL-1, #94296, Cell Signaling), proto-oncogene serine/threonine-protein kinase Pim-1 (PIM-1, #54523, Cell Signaling), and β-actin (#ab8227, Abcam). After rinsing with Tris buffer saline containing TBST, the PVDF membranes were incubated with goat anti-rabbit secondary antibody (1:2,000, #6721, Abcam) for 1 h. The protein bands were detected

and the density of bands was quantified using the AlphaImager 2000 Imaging System (Alpha Innotech, USA).

Transfection of the JAK2 overexpression plasmid

The human *JAK2* coding sequences (NM-004972.3) were amplified and cloned to produce the JAK2 overexpression plasmid, as previously reported [16]. The MCF-7 cells were seeded into 24-well plates and incubated overnight, and then transfected with the JAK2 overexpression vector or the empty vector using Genjuice Transfection Reagent (MilliporeSigma, Burlington, USA). Twenty-four hours later, *JAK2* gene expression was quantified using real-time PCR to determine the transfection efficiency using the primers: 5'-GGGAGGTGGTCGCTGTAAAA-3' (forward) and 5'-ACCAGCACTGTAGCACACTC-3' (reverse).

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA), and reverse-transcribed to cDNA. Real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystem, Foster City, USA). The relative expression level of *JAK2* was normalized to *U6*. Then, the cells transfected with the JAK2 overexpression vector or the empty vector were treated with 0 or 50 μg/mL APS for 96 h before the MTT assay.

Statistical analysis

Experiments were performed in triplicate and data are shown as mean ± standard deviation. One-way analysis of variance (ANOVA) was performed using SPSS (version 24.0). A value of $p < 0.05$ was considered to be statistically different.

RESULTS

APS induced apoptosis in MCF-7 breast cancer cells

To investigate the effects of APS on cell proliferation and apoptosis, MCF-7 cells were treated with APS (0, 10, 30, 50, or 100 μg/mL APS) for 4 days. MTT assay showed that APS significantly decreased cell proliferation in a concentration-dependent manner (Figure 1 A). Similarly, compared to the control group, treatment with APS induced cell apoptosis as demonstrated by a higher percentage of annexin V-positive apoptotic cells and a lower number of live cells. (Figure 1 B). The expression of proliferation- and apoptosis-related proteins, including cleaved caspase-3, BCL-2, BAX and cleaved poly ADP ribose polymerase (PARP) [17], were also detected using western blotting. Treatment with 10 – 100 μg/mL APS inhibited BCL-2 expression, but increased the expression levels of BAX, cleaved caspase-3, and cleaved PARP (Figure 1 C). Thus, APS reduced cell proliferation and promoted cell apoptosis in MCF-7 cells.

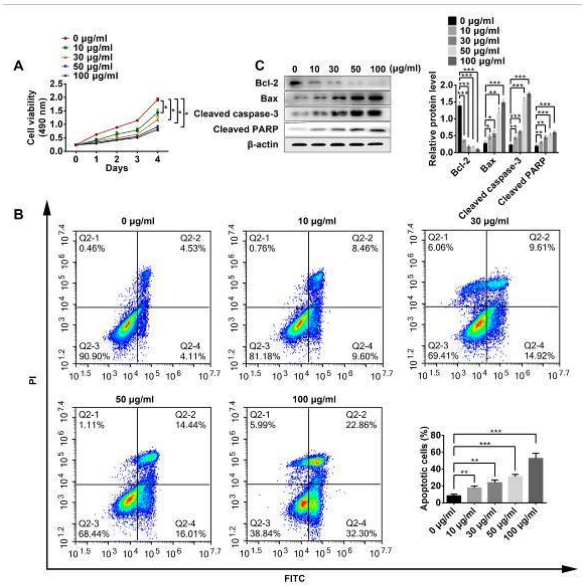

Figure 1: Effect of APS on MCF-7 cell apoptosis. MCF-7 cells were treated with 0, 10, 30, 50, or 100 μg/mL APS for 96 h. Cell proliferation was evaluated using the MTT assay (A). Cell apoptosis was evaluated using flow cytometry (B). Expression levels of apoptosis-related proteins were detected by western blot (C). Asterisks indicate statistical significance; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared to 0μg/mL APS; one-way ANOVA test was used

APS inhibited JAK/STAT activation in MCF-7 breast cancer cells

Compared to non-treated controls, APS-treated cells showed significantly lower levels of phosphorylated JAK2 and phosphorylated STAT3. Myeloid cell leukemia-1 (MCL-1) and proto-oncogene serine/threonine-protein kinase (PIM-1), downstream targets of the JAK/STAT pathway, exert anti-apoptotic effects on breast cancer cells [19,20]. Treatment with APS also inhibited expression of MCL-1 and PIM-1 (Figure 2). Thus, APS treatment inactivated the JAK/STAT pathway and downregulated the expression of its target genes in MCF-7 cells.

Inhibition of the JAK/STAT pathway promoted apoptosis in MCF-7 cells

For further analysis, MCF-7 cells were treated with ruxolitinib, a classical JAK inhibitor [18], for 4 days. The results showed ruxolitinib significantly

inhibited the cell viability (Figure 3A). A higher percentage of apoptotic cells was found in ruxolitinib-treated cells, indicating that ruxolitinib significantly prompted cell apoptosis (Figure 3B). Upon treatment with ruxolitinib, the protein expressions of phosphorylated JAK2, phosphorylated STAT3, BCL-2, MCL-1 and PIM-1 were downregulated as compared to the control. However, the protein expressions of BAX, cleaved caspase-3, and cleaved PARP were increased in ruxolitinib-treated MCF-7 cells as compared to the control (Figure 3C). These data showed that inhibition of the JAK/STAT pathway induced apoptosis of MCF-7 cells.

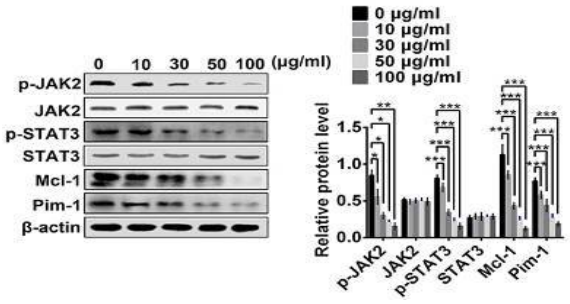

Figure 2: Effect of APS on the JAK/STAT pathway in MCF-7 cells. Cells were treated with 0, 10, 30, 50, or 100 μg/mL APS for 96 h, and then cell lysates were collected. Protein expressions were assessed using western blot. Asterisks indicate statistical significance; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to 0μg/mL APS; one-way ANOVA test was used

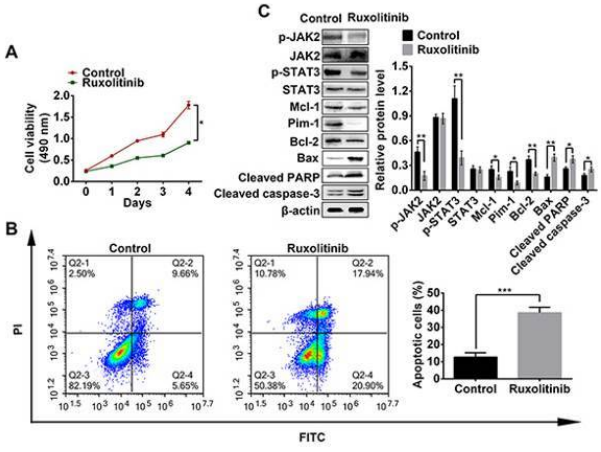

Figure 3: Effect of JAK inhibitor on JAK/STAT activation and MCF-7 cell apoptosis. Cells were treated with or without 20 μM ruxolitinib for 96 h. Cell proliferation was evaluated using the MTT assay (A). Cell apoptosis was evaluated using flow cytometry (B). Expressions of proteins were measured using western blot and normalized to β-actin (C). Asterisks indicate statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control group, one-way ANOVA test was used

APS stimulated cell apoptosis via inhibition of JAK/STAT activation in MCF-7 breast cancer cells

MCF-7 cells were transfected with JAK2 overexpression plasmid to determine whether APS regulated cell apoptosis in MCF-7 cells via the JAK/STAT pathway. The mRNA and protein expressions of JAK2 were significantly higher in cells that transfected with JAK2 overexpression plasmid than control group (Figure 4 A). JAK2 overexpression significantly increased cell viability than the control group. Though APS significantly decreased cell viability, JAK2 overexpression reversed these results (Figure 4 B). Western blot analysis showed that JAK2 overexpression-induced upregulation of p-JAK2 and p-STAT3 was significantly suppressed by APS treatment (Figure 4 C). The cell apoptosis assay showed that JAK2 overexpression inhibited cell apoptosis as compared to the control. After APS treatment, JAK2-transfected cells showed significantly more apoptotic cells than that without APS treatment. (Figure 4 D). Taken together, JAK2 overexpression increased viability and suppressed apoptosis in MCF-7 cells, and these effects were reversed by APS via inhibition of the JAK/STAT pathway.

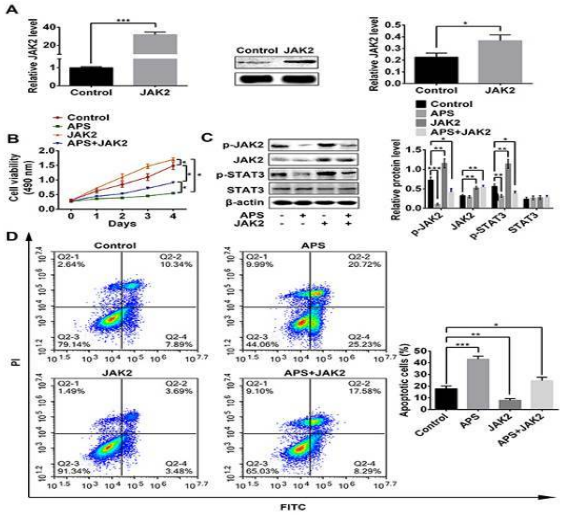

Figure 4: JAK2 overexpression-induced suppression of cell apoptosis was reversed by APS via inhibition of the JAK/STAT pathway. MCF-7 cells were transfected with JAK2 overexpression plasmid (JAK2) or the empty vector (control). JAK2 mRNA and protein expression were evaluated 24 h after transfection by RT-PCR and western blot (A). JAK2-transfected cells and control cells were treated with or without 50 μg/mL APS for 96 h. Cell viability was evaluated using the MTT assay (B). Expression proteins were measured using western blot (C). Cell apoptosis was evaluated by flow cytometry (D). Asterisks indicate statistical significance; *p < 0.05, **p < 0.01, ***p < 0.001 as compared to control group; one-way ANOVA test was used

DISCUSSION

The progression of cancer involves the dysregulation of programmed cell death. Thus, apoptosis is a potential target of anticancer strategies [19]. The anti-tumor effect of polysaccharides extracted from *Angelica sinensis* has been widely explored in various cancers, including hepatoma, cervical cancer, and breast cancer [11,12,20] Consistently, this study showed that APS induced apoptosis in human MCF-7 breast cancer cells.

Janus kinase 2 (JAK2) is the key kinase involved in the STAT signaling pathway. Phosphorylated JAK2 triggers the activation of STAT3, followed by the dimerization, translocation, and DNA-binding of STAT3 to JAK2/STAT3 target genes [21]. Persistent activation of STAT3 has been detected in many tumors, and interruption of STAT3 activity resulted in apoptosis of cultured tumor cells [22]. In this study, high levels of phosphorylated STAT3 and JAK2 were observed in MCF-7 cells, whereas these results were reversed by APS treatment.

Phosphorylation of STAT3 leads to transcriptional activation of its downstream target genes which regulate cell survival and proliferation, such as *MCL-1* and *PIM-1* [23,24]. MCL-1 is an anti-apoptotic member of the BCL-2 family, and MCL-1 impedes apoptosis by binding and sequestering the pro-apoptotic proteins in BCL-2 family [25]. In human breast cancer cells, APS promoted mitochondrial-dependent apoptosis by downregulating MCL-1 protein expression [12]. PIM-1 is a member of the PIM kinase family and shown to prevent mitochondrial-mediated apoptosis in triple-negative breast cancer cells lines [26]. In contrast to the activation of JAK/STAT, downregulation of STAT3 activity were correlated to reduced protein expressions of BCL-2 and MCL-1 [27,28]. Consistent with previous findings, inhibition of the JAK2/STAT3 signaling pathway by ruxolitinib, a JAK inhibitor, significantly reduced expressions of proliferation-related proteins.

BAX is a member of the BCL-2 gene family that acts as a pro-apoptotic regulator [29]. It has been shown that inhibition of the JAK/STAT3 signaling pathway led to an increase in BAX expression in a lung adenocarcinoma cell line [30]. Inhibition of STAT3 in human osteosarcoma cells induced apoptosis as demonstrated by the cleavage of PARP and caspase-3 [31]. It has also been shown that APS stimulate apoptosis in breast cancer cells by upregulating PARP and caspase-3 [12]. These findings indicate that the loss of JAK2/STAT3 activity increases expression of pro-apoptotic proteins. In this study, treatment with the JAK inhibitor increased expression of BAX, cleaved PARP, and cleaved caspase-3. Similar to cells treated with ruxolitinib, treatment with APS led to reduced protein expressions of BCL-2, MCL-1, and PIM-1 and enhanced expression of BAX, cleaved PARP, and cleaved caspase-3. Moreover, the anti-apoptotic effect of JAK2 overexpression was weakened in the presence of APS.

CONCLUSION

The current study revealed that polysaccharide isolated from *Angelica sinensis* promotes apoptosis in breast cancer cells via inhibition of the JAK2/STAT3 signaling pathway. It provides a novel understanding of the pro-apoptotic mechanism of APS *in vitro* and insight on the development of APS as a potential therapeutic agent for breast cancer.

DECLARATIONS

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Zhaoyuan Fu and Yingdong Li designed all the experiments and revised the manuscript. Suisheng Yang and Chengxu Yang formed the experiments. Rongrong Zhao, Huan Guo and Huiping Wei wrote the manuscript.

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