Safflower polysaccharide inhibits the development of tongue squamous cell carcinoma

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Abstract

Background: Safflower polysaccharide (SPS) is one of the most important active components of safflower (Carthamus tinctorius L.), which has been confirmed to have the immune-regulatory function and antitumor effect. This study aimed to explore the effects of safflower polysaccharide (SPS) on tongue squamous cell carcinoma (TSCC).

Methods: HN-6 cells were treated with 5 μg/mL cisplatin and various concentrations of SPS (0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, and 1.28 mg/mL), and cell proliferation was measured. After treatment with 5 μg/mL cisplatin and 0.64 mg/mL SPS, the induction of apoptosis and the protein and mRNA expression of Bax, Bcl-2, COX-2, and cleaved caspase-3 in HN-6 cells were quantified. In addition, HN-6 cells were implanted into mice to establish an in vivo tumor xenograft model. Animals were randomly assigned to three groups: SPS treatment, cisplatin treatment, and the model group (no treatment). The body weight, tumor volume, and tumor weight were measured, and the expression of the above molecules was determined.

Results: SPS treatment (0.02–0.64 mg/mL) for 24–72 h inhibited HN-6 cell proliferation. In addition, 0.64 mg/mL SPS markedly induced apoptosis in HN-6 cells and arrested the cell cycle at the G0/G1 phase. Compared with the control group, the expression of Bcl-2 and COX-2 was markedly reduced by SPS treatment, whereas the expression of Bax and cleaved caspase-3 was increased. Moreover, SPS significantly inhibited the growth of the tumor xenograft, with similar changes in the expression of Bcl-2, COX-2, Bax, and cleaved caspase-3 in the tumor xenograft to the in vitro analysis.

Conclusions: Our results indicated that SPS may inhibit TSCC development through regulation of Bcl-2, COX-2, Bax, and cleaved caspase-3 expression.

Keywords: Safflower polysaccharide, Tongue squamous cell carcinoma, Apoptosis, Bcl-2, COX-2, Bax, Cleaved caspase-3

Background

Tongue squamous cell carcinoma (TSCC) is a primary malignant tumor of the tongue, with the highest incidence rate (approximately 39.95%) among oral cancers [1]. With early detection, TSCC can be cured with proper treatment; however, after tumor metastasis, the 5-year overall survival rate is below 50% [2–4]. Therefore, the research and development of effective drugs and methods for the treatment of TSCC would have far-reaching consequences.

Safflower (Carthamus tinctorius L.) is a herbaceous plant of the Asteraceae family, containing various active constituents, including flavonoids, quinochalcones, alkaloids, and safflower polysaccharides (SPS) [5]. Safflower exerts various biological effects, including antioxidant [6], anti-inflammatory [7], and antibacterial [8] activities, and is reported to be beneficial for the improvement of acute cerebral infarction [9] and ischemic stroke [10]. SPS is one of the most important active components of safflower, and accumulating evidences have supported the immuno-regulatory function and antitumor effect of SPS [11–13]. In breast cancer, SPS is shown to inhibit the MCF-7 cell proliferation and metastasis [14]. SPS is also found to inhibit proliferation of human hepatic cancer SMMC-7721 cells through the regulation of the expression...
of cell cycle-related genes [15]. Moreover, SPS is confirmed to affect cell growth and apoptosis in non-small cell lung cancer [16], gastric cancer [17–19], and colorectal cancer [20]. However, the role of SPS in the development of TSCC remains unexplored.

In this study, we detected the effect of SPS on HN-6 cell proliferation and apoptosis. Moreover, HN-6 cells were implanted into mice to establish an in vivo tumor xenograft model for the assessment of the effect of SPS on tumor growth. The present study investigated the roles and regulatory mechanism of SPS in TSCC to provide new strategies for TSCC therapy.

Methods
SPS preparation
The crude drug containing SPS was purchased from Shiyitang Co., Ltd. (Harbin, PR China), and voucher specimens (No. HLJ-2015008) were deposited at College of Basic Medical Science, Heilongjiang University of Chinese Medicine. The crude drug was dried at 60 °C in a vacuum oven for 24 h and extracted four times in boiling water with agitation for 1 h. The extracts were filtered, concentrated, and precipitated with four volumes of 95% ethanol at 4 °C for 24 h. The mixture was centrifuged, and the sediment was dried at 60 °C in a vacuum oven. The protein contaminants were extracted with Sevage reagent (a 4:1 (v/v) mixture of chloroform to n-butyl alcohol) and removed by centrifugation; this process was repeated 10 times. The water phase was then precipitated in four volumes of 95% ethanol. The sediments were oven-dried at 60 °C to produce SPS, a light-yellow powder, at yield of 0.382% (w/w). SPS was composed of d-glucose in a weight ratio of 97.06%.

Cell culture
The TSCC cell line, HN-6, was obtained from the Laboratory of Oncological Biology of the Ninth Hospital Affiliated to Shanghai Jiao Tong University (Shanghai, PR China). HN-6 cells were then maintained in Dulbecco’s modified Eagle medium (DMEM; pH 7.2; Sigma-Aldrich, Shanghai, PR China) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Shanghai, PR China) in a humidified atmosphere of 5% CO2. HN-6 cells were seeded in a 96-well plate (6.0 × 106 cells/well) and cultured in DMEM supplemented with 10% FBS at 37 °C for 48 h. Then, 5 μg/mL cisplatin and 0.64 mg/mL SPS were added separately and incubated with the cells for 24, 48, and 72 h. After this, 5 μL dye mixture (500 mg/mL AO and 500 mg/mL EB in distilled water) was added to each well. Cell apoptosis was then examined by an inverted phase-contrast microscope (Olympus IX70, Hamburg, Germany) at × 400 magnification.

Cell cycle analysis
Cell cycle analysis was performed using a Cell Cycle Detection Kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, JiangSu, China). Briefly, HN-6 cells were seeded in a 6-well plate and incubated with DMEM supplemented with 10% FBS at 37 °C for 48 h. Then, 5 μg/mL cisplatin and 0.64 mg/mL SPS were added to each well and incubated with the cells for 48 h. The cells were harvested, fixed with 75% ice-cold ethanol, and stained with 400 μL propidium iodide (PI) for 45 min in the dark. The cell cycle analysis after different drug treatments was then conducted using a FACSCalibur flow cytometer (BD, USA).

qRT-PCR
Total RNA was extracted using the Trizol kit (Invitrogen, Carlsbad, CA, USA), reverse transcription was then performed using an M-MLV RTase kit (Promega, USA), and qRT-PCR was then performed using One Step SYBR PrimeScript RT-PCR Kit (Takara, PR China) and an ABI-7500 PCR machine (Applied Biosystems, USA). The amplification procedure comprised 95 °C for 15 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The forward and reverse primer sequences, respectively, for the amplification of targets were as follows: Bax, 5′-GGCCCTTTTCCTCAAGGCTT-3′ and 5′-GGAAGAATGACCTCTCGGGGGG-3′; Bcl-2, 5′-CTTTGAGTTGCGTGGGTCA-3′ and 5′-GGGCCCTACAGTTCCACAAA-3′; COX-2, 5′-TTTGATTTCCTTGCCCCG-3′ and 5′-GGGA GATACATCTCTCTCAAT-3′; cleaved caspase-3, 5′-AGCAATATGAATGGCCGTAG-3′ and 5′-GTAT GAGAAATGGGGTGTAG-3′; and GAPDH, 5′-CGCT GAGTACGGTCGAGGTC-3′ and 5′-GCTGATGATCTTGAGGCTGTC-3′. The relative expression of these
target genes were normalized to GAPDH and then calculated using the $2^{-\Delta\Delta CT}$ method.

**Western blot analysis**

Total protein was extracted in lysis buffer containing PMSF (Pierce; Rockford, IL). The proteins were separated in a 10% SDS-PAGE and immunoblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, USA). Non-specific binding to the membranes was blocked by incubation with 5% nonfat dried milk for 1–2 h, and the membranes were probed separately with rabbit anti-human Bax, Bcl-2, COX-2, cleaved caspase-3, and GAPDH antibodies (1:100, Invitrogen) overnight at 4 °C, followed incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10000, Invitrogen) for 1 h; GAPDH was used as the internal control. The protein bands were visualized by the application of 4-chloronaphthol (Sigma-Aldrich) and analyzed by Gel-Pro 4.0 software (Media Cybernetics, Inc., USA).

**In vivo tumor xenograft model**

Twenty female BALB/cnu/nu nude mice (4–6 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). These mice were housed in a room at constant temperature (27 °C ± 1 °C) and humidity (50% ± 10%) and a 12-h light/dark cycle.

| Group | Concentration (mg/mL) | OD value (24 h) | IR% | OD value (48 h) | IR% | OD value (72 h) | IR% |
|-------|------------------------|----------------|-----|----------------|-----|----------------|-----|
| SPS   | 0.02                   | 0.816 ± 0.028  | 1.73| 0.798 ± 0.033* | 10.82| 0.649 ± 0.023* | 27.71|
|       | 0.04                   | 0.811 ± 0.054  | 4.74| 0.760 ± 0.022* | 16.24| 0.643 ± 0.036* | 31.43|
|       | 0.08                   | 0.772 ± 0.033  | 12.41| 0.659 ± 0.012* | 20.18| 0.622 ± 0.019* | 35.33|
|       | 0.16                   | 0.677 ± 0.028  | 18.13| 0.619 ± 0.015* | 35.41| 0.582 ± 0.052* | 38.62|
|       | 0.32                   | 0.628 ± 0.056  | 33.33| 0.496 ± 0.033* | 42.53| 0.321 ± 0.031* | 47.78|
|       | 0.64                   | 0.488 ± 0.027  | 46.72| 0.309 ± 0.031* | 51.29| 0.266 ± 0.058** | 64.95|
|       | 1.28                   | 0.53 ± 0.081   | 42.63| 0.451 ± 0.042* | 48.12| 0.306 ± 0.036* | 56.32|
| Cisplatin | $5 \times 10^{-3}$ | 0.461 ± 0.034  | 58.66| 0.283 ± 0.011** | 75.59| 0.308 ± 0.032** | 72.09|
| Control | 0.00                   | 0.822 ± 0.021  | -   | 0.850 ± 0.023  | -   | 0.996 ± 0.012  | -   |

SPS safflower polysaccharide, OD optical density, IR inhibitory rate. *P < 0.05 and **P < 0.01

Fig. 1 Acridine orange/ethidium bromide (AO/EB) double staining (× 400) was used to detect the apoptosis of HN-6 cells in the control-, 5 μg/mL cisplatin-, and 0.64 mg/mL SPS-treated groups.
cycle for 1 week. HN-6 cells (4 × 10^7) were subcutaneously injected into the flanks of mice to establish the in vivo tumor xenograft model. When the volumes of the xenografts reached 100–300 mm^3, the animals were randomly divided into three groups: SPS (n = 8), cisplatin (n = 8), and model (n = 4). Throughout the 15 days of treatment, the mice in the SPS group were injected with 40 mg/kg SPS once a day, and the mice in cisplatin and model groups were injected with 0.8 mL/day cisplatin and normal saline every 3 days, respectively. The animal experiments were approved by the Animal Care and Use Committee of China Medical University (Taichung, Taiwan).

During treatment, the body weight of the tumor-bearing mice and the tumor size (length and width) were measured every 3 days. The tumor volume was estimated from the following equation: tumor volume = 0.5ab^2, where a is the length of the tumor and b is the width of the tumor. The animals were sacrificed at the end of the study, and the tumors were removed and weighed. Tumor xenografts were used for the analysis of the expression of COX-2, Bcl-2, Bax, and cleaved caspase-3 by qRT-PCR and western blot analysis.

Statistical analysis
All measurement data from multiple experiments were presented as the mean ± standard deviation. One-way ANOVA was performed to analyze the significance of differences among groups, followed by a Tukey post hoc test for further between-group comparisons. Statistical software SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was applied, and statistical significance was accepted at P < 0.05.

Results

SPS inhibited HN-6 cell proliferation
The effect of SPS on HN-6 cell proliferation was evaluated by a CCK8 assay. The results indicated that SPS inhibited HN-6 cell proliferation in a dose- and time-dependent manner within a certain dose range (0.02–0.64 mg/mL) and time (24–72 h) (Table 1). Among these concentrations of SPS (0.02–1.28 mg/mL), 0.64 mg/mL SPS exhibited the strongest IR on HN-6 cell proliferation at different time points (Table 1), and this concentration was therefore selected for subsequent experiments.

SPS induced the apoptosis of HN-6 cells
AO/EB double staining showed that the morphologies of HN-6 cells in the control group had intact structure and green-stained nuclei (Fig. 1). After treatment with cisplatin or 0.64 mg/mL SPS, shrinkage, chromatin condensation, membrane blebbing, and the formation of apoptotic bodies were identified in HN-6 cells (Fig. 1), indicating that SPS induced apoptosis in HN-6 cells.

SPS arrested cell cycle in the G0/G1 phase
The effect of SPS on the cell cycle was also examined. Compared with the control group, the percentage of HN-6 cells in the G0/G1 phase was significantly increased after treatment with 0.64 mg/mL SPS for 48 h, whereas the percentage of HN-6 cells in the G2/M phase was markedly decreased (Table 2), which indicated that SPS arrested the cell cycle of HN-6 cells in the G0/G1 phase. The percentage of HN-6 cells in different cell cycle stages was not significantly different in the control and cisplatin groups (Table 2).

Analysis of mRNA and protein expression of Bcl-2, COX-2, Bax, and cleaved caspase-3 in HN-6 cells after treatment
To investigate the regulatory mechanism of SPS, the mRNA and protein expression of Bcl-2, COX-2, Bax, and cleaved caspase-3 were detected. The results showed that, compared with the control group, the expression of Bcl-2 and COX-2 were downregulated, while the expression of Bax and cleaved caspase-3 were upregulated.

Table 2 The effect of 0.64 mg/mL SPS on HN-6 cell cycle

| Groups  | G0/G1 (%) | S   | G2/M |
|---------|-----------|-----|------|
| Control | 10.71     | 52.42 | 36.87 |
| SPS     | 25.76*    | 50.49 | 23.75* |
| Cisplatin | 14.51   | 47.99 | 37.50 |

SPS safflower polysaccharide. *P < 0.05

Table 3 The relative expression of target mRNAs in the control, cisplatin, and 0.64 mg/mL SPS groups

| Groups  | Bcl-2   | COX-2  | Bax     | Cleaved caspase-3 |
|---------|---------|--------|---------|------------------|
| Control (24 h) | 0.713 ± 0.031 | 0.638 ± 0.048 | 0.185 ± 0.043 | 0.262 ± 0.081 |
| Control (48 h) | 0.720 ± 0.0196 | 0.592 ± 0.005 | 0.166 ± 0.121 | 0.321 ± 0.021 |
| Control (72 h) | 0.693 ± 0.232 | 0.799 ± 0.923 | 0.203 ± 0.056 | 0.301 ± 0.044 |
| Cisplatin (24 h) | 0.585 ± 0.055 | 0.513 ± 0.023* | 0.481 ± 0.011* | 0.477 ± 0.021* |
| Cisplatin (48 h) | 0.451 ± 0.060* | 0.416 ± 0.019* | 0.566 ± 0.035* | 0.681 ± 0.046* |
| Cisplatin (72 h) | 0.281 ± 0.026* | 0.264 ± 0.032* | 0.762 ± 0.055* | 0.872 ± 0.065* |
| SPS (24 h) | 0.423 ± 0.055* | 0.408 ± 0.023* | 0.314 ± 0.011* | 0.585 ± 0.034* |
| SPS (48 h) | 0.345 ± 0.060* | 0.222 ± 0.019* | 0.426 ± 0.035 | 0.822 ± 0.081* |
| SPS (72 h) | 0.201 ± 0.026* | 0.118 ± 0.032* | 0.582 ± 0.055* | 1.031 ± 0.092* |

SPS safflower polysaccharide. *P < 0.05 compared with the control group
and COX-2 mRNA and protein was significantly decreased after SPS or cisplatin treatment in a time-dependent manner, whereas that of Bax and cleaved caspase-3 was obviously increased (Tables 3 and 4).

**SPS inhibited the growth of tumor xenograft**

The in vivo tumor xenograft model was established to explore the effects of SPS. The tumor weights in the model, SPS, and cisplatin groups were 2.236 ± 0.063, 1.145 ± 0.210, and 0.963 ± 0.049 g, respectively. Moreover, compared with the model group, the tumor volume of the SPS and cisplatin groups were markedly decreased after 2 weeks of intervention \( (P < 0.05, \text{Fig. 2}) \). However, there were no significant differences in the body weight of mice in the different groups (data not shown).

**Table 4** The relative expression of the target proteins in the control, cisplatin, and 0.64 mg/mL SPS groups

| Groups      | Bcl-2     | COX-2     | Bax      | Cleaved caspase-3 |
|-------------|-----------|-----------|----------|-------------------|
| Control (24 h) | 0.723 ± 0.121 | 0.918 ± 0.095 | 0.282 ± 0.164 | 0.464 ± 0.091 |
| Control (48 h) | 0.719 ± 0.196 | 0.922 ± 0.132 | 0.283 ± 0.053 | 0.460 ± 0.021 |
| Control (72 h) | 0.733 ± 0.198 | 0.899 ± 0.210 | 0.290 ± 0.026 | 0.465 ± 0.018 |
| Cisplatin (24 h) | 0.442 ± 0.113* | 0.661 ± 0.066* | 0.676 ± 0.103* | 0.679 ± 0.093* |
| Cisplatin (48 h) | 0.393 ± 0.045* | 0.458 ± 0.072* | 0.867 ± 0.064* | 0.863 ± 0.123* |
| Cisplatin (72 h) | 0.107 ± 0.062 | 0.237 ± 0.069 | 0.916 ± 0.093* | 0.991 ± 0.133* |
| SPS (24 h) | 0.513 ± 0.101* | 0.795 ± 0.135* | 0.740 ± 0.0858 | 0.602 ± 0.076* |
| SPS (48 h) | 0.392 ± 0.083* | 0.590 ± 0.099* | 0.920 ± 0.083* | 0.764 ± 0.063* |
| SPS (72 h) | 0.206 ± 0.106* | 0.435 ± 0.021* | 1.832 ± 0.925* | 0.948 ± 0.089* |

*SPS safflower polysaccharide. *\( P < 0.05 \) compared with the control group

Analysis of mRNA and protein expression of Bcl-2, COX-2, Bax, and cleaved caspase-3 in tumor xenograft model after different treatments

Consistent with the expression changes in HN-6 cells after different treatment, Bcl-2 and COX-2 expression in the tumor xenografts from the SPS or cisplatin groups was significantly lower than those from the model group, and the expression of Bax and cleaved caspase-3 was increased (Fig. 3).

**Discussion**

The present study illustrated that SPS markedly inhibited HN-6 cell proliferation, induced apoptosis, and arrested the cell cycle of HC-6 cells in the G0/G1 phase. Bcl-2 and COX-2 expression was significantly decreased after SPS treatment, whereas Bax and cleaved caspase-3 was significantly increased. Moreover, SPS significantly inhibited growth of the in vivo tumor xenografts, and the changes in the expression of the above molecules in tumor xenograft model after SPS intervention were consistent with previous results.

Apoptosis is an important mechanism involved in cancer progression, involving a series of active death process after the stimulation of many types of death signals [21, 22]. Bcl-2 families, such as Bax and Bcl-2, are regarded as a key mediator of cell apoptosis [23]. The ratio of Bcl-2 to Bax is shown to affect cellular sensitivity to the apoptotic signals. A previous study has confirmed that the mechanism of cantharidin in the promotion of apoptosis in TSCC may be associated with the suppression of the Bcl-2/Bax signaling pathway [24]. Moreover, Bcl-2 inhibition and Bax activation are thought to be promising approaches for cancer therapy [25, 26]. Notably, safflower injection resulted in an increase in the Bax/Bcl-2 ratio [27], which prompted us to speculate that SPS may inhibit TSCC development through the regulation of the Bcl-2/Bax expression ratio. In addition, caspase-3 is also considered to be a key mediator of aerobic metabolism and appears to play an important role in the death signals of cells. caspase-3 is also considered to be a key mediator of aerobic metabolism and appears to play an important role in the death signals of cells.
mitochondrial apoptosis [28]. Cleaved caspase-3 can induce apoptosis through blocking the contact between the cell and its surroundings [29, 30]. Importantly, the activation of caspase-3 can induce apoptosis in TSCC [31]. In this study, we found that SPS induced apoptosis in HN-6 cells. Moreover, the Bax/Bcl-2 ratio and the expression of cleaved caspase-3 were increased after SPS treatment. Therefore, SPS may promote cell apoptosis in TSCC through an increase in the Bax/Bcl-2 ratio and the expression of cleaved caspase-3.

Furthermore, we also found that the expression of COX-2 was significantly decreased after SPS treatment. COX-2 is an inducible enzyme implicated in the transformation of arachidonic acid to prostaglandin and other eicosanoids [32]. An accumulation of evidence supports the overexpression of COX-2 in various tumor tissues and cells and a close relationship with tumor development [33]. Cao et al. demonstrated that miR-26b regulated cell proliferation and metastasis in TSCC through the regulation of COX-2 [34]. Moreover, COX-2 inhibition suppresses angiogenesis and tumor growth, potentiating antiangiogenic cancer therapy [35]. Consistent with a previous study, in which the dried aqueous extracts of safflower petal attenuated COX-2 protein expression and protected against lipopolysaccharide-induced inflammation [36], we found that SPS treatment resulted in a decrease in the expression of COX-2. Given the key role of COX-2 in tumor development, we speculated that SPS may inhibit TSCC development through a decrease in the expression of COX-2.

Conclusions
In conclusion, our results indicated that SPS may inhibit TSCC development through the regulation of the expression of Bcl-2, COX-2, Bax, and cleaved caspase-3. However, as this study is preliminary, further experiments are required to explore the possible mechanism of SPS in the prevention of TSCC development.

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Authors’ contributions
HZ contributed to the conception and design of the research. RW contributed to the acquisition of the data. CZ contributed to the analysis and interpretation of the data. YZ contributed to the statistical analysis. JY helped in obtaining the funding. XL helped in drafting the manuscript. SZ contributed to the revision of the manuscript for important intellectual content. All authors gave final approval of the manuscript.

Ethics approval and consent to participate
This study was approved by Ethics Committee of The First Affiliated Hospital of Harbin Medical University, Heilongjiang University of Chinese Medicine, and Guiyang University of Chinese Medicine.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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