Postprint: Shijianchuan Induces Ferroptosis to Inhibit Esophageal Carcinogenesis and Progression in Mice

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Abstract

Background: Esophageal cancer is a common malignant tumor of the digestive tract with high incidence and mortality rates both in China and worldwide. As a traditional Chinese medicine, Shijianchuan (SJC) is commonly employed in the treatment of esophageal cancer for its heat-clearing and detoxifying, blood-activating and analgesic effects. Pharmacological experimental studies have demonstrated that SJC possesses anticancer properties and can effectively treat various malignant tumors.

Objective: To investigate the role and mechanism of SJC in inhibiting the occurrence and development of orthotopic esophageal cancer in C57 mice based on ferroptosis.

Methods: From February 2022 to February 2023, 90 SPF-grade female C57BL/6 mice were selected and randomly divided into a control group (Control group, n=15), a 4NQO-only carcinogenesis group (4NQO group, n=25), a 4NQO + low-dose SJC group [4NQO/SJC (91 mg) group, n=25], and a 4NQO + high-dose SJC group [4NQO/SJC (182 mg) group, n=25]. An orthotopic esophageal cancer model in C57 mice was established using 4NQO induction. Mouse activity was observed, and their mental status and food/water intake were recorded. Body weight was measured and documented for each group every 8 weeks. At 32 weeks, hematoxylin-eosin (HE) staining and pathological analysis of esophageal tissues were performed. The contents of Fe2+, glutathione (GSH), and malondialdehyde (MDA) in esophageal tissues were measured. Western blotting was employed to detect the protein expression levels of nuclear receptor coactivator 4 (NCOA4) and glutathione peroxidase 4 (GPX4) in mouse esophageal tissues. The Kaplan-Meier method was used to plot mouse survival curves, and the Breslow test was applied for survival curve comparisons.

Results: At 8, 16, 24, and 32 weeks after modeling, the body weight of mice in the 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups was lower than that in the Control group; at 32 weeks, the body weight of mice in the 4NQO/SJC (91 mg) and 4NQO/SJC (182 mg) groups was higher than that in the 4NQO group ($P < 0.05$). Breslow test results showed a statistically significant difference in survival curves among the four groups of mice ($2 = 9.907$, $P = 0.019$). HE staining results revealed that the esophageal epithelial tissue in the 4NQO group exhibited abnormal hyperplasia, disordered cell arrangement, and abnormal pathological changes such as keratin pearls; compared with the 4NQO group, pathological changes in the esophageal epithelial tissue were significantly improved in the 4NQO/SJC (91 mg) and 4NQO/SJC (182 mg) groups. The Fe2+ and MDA levels in the 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups were lower than those in the Control group, while GSH was higher than that in the Control group; the Fe2+ and MDA levels in the 4NQO/SJC (91 mg) and 4NQO/SJC (182 mg) groups were higher than those in the 4NQO group, while GSH was lower than that in the 4NQO group; the Fe2+ and MDA levels in the 4NQO/SJC (182 mg) group were higher than those in the 4NQO/SJC (91 mg) group, while GSH was lower than that in the 4NQO/SJC (91 mg) group ($P < 0.05$). NCOA4 in the 4NQO group was lower than that in the Control group, 4NQO/SJC (91 mg) group, and 4NQO/SJC (182 mg) group, while GPX4 was higher than that in the Control group, 4NQO/SJC (91 mg) group, and 4NQO/SJC (182 mg) group; GPX4 in the 4NQO/SJC (91 mg) and 4NQO/SJC (182 mg) groups was higher than that in the Control group.

Conclusion: SJC can intervene in the occurrence and development of esophageal cancer, and its mechanism may be related to NCOA4-mediated ferritinophagy.

Full Text

Study on the Inhibition of Esophageal Carcinoma Development in Mice by Salvia chinensis through Induced Ferroptosis

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Abstract

Background: Esophageal carcinoma is a common malignant tumor of the gastrointestinal tract in China and globally, with high incidence and mortality rates. Salvia chinensis (SJC), a traditional Chinese medicine known for its heat-clearing, detoxifying, blood circulation-promoting, and analgesic effects, is commonly used in esophageal cancer treatment. Pharmacological studies have demonstrated that SJC possesses anticancer properties and can effectively treat various malignant tumors.

Objective: To investigate the effect and mechanism of SJC in inhibiting the development of carcinoma in situ in C57 mouse esophageal cancer based on ferroptosis.

Methods: Between February 2022 and February 2023, ninety SPF-grade female C57BL/6 mice were randomly divided into four groups: control group (Control, n=15), 4NQO-induced cancer group (4NQO, n=25), low-dose SJC group [4NQO/SJC (91 mg), n=25], and high-dose SJC group [4NQO/SJC (182 mg), n=25]. An orthotopic esophageal cancer model in C57 mice was established using 4NQO induction. Mouse activity, mental status, and food and water intake were observed and recorded, with body weight measured every 8 weeks. After 32 weeks, hematoxylin-eosin (HE) staining and pathological analysis of esophageal tissues were performed. The contents of Fe^{2+}, glutathione (GSH), and malondialdehyde (MDA) in esophageal tissues were measured, and protein expression levels of nuclear receptor coactivator 4 (NCOA4) and glutathione peroxidase 4 (GPX4) were detected by western blot. Survival curves were generated using the Kaplan-Meier method and compared using the Breslow test.

Results: At 8, 16, 24, and 32 weeks of modeling, body weight in the 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups was lower than in the Control group. At 32 weeks, body weight in both SJC-treated groups was significantly higher than in the 4NQO group ($P<0.05$). Breslow test results showed statistically significant differences in survival curves among the four groups ($^{2}=9.907$, $P=0.019$). HE staining revealed abnormal proliferation, disordered cell arrangement, and pathological changes such as keratin pearls in the 4NQO group esophageal epithelium. Compared with the 4NQO group, pathological changes in the 4NQO/SJC (91 mg) and 4NQO/SJC (182 mg) groups were significantly improved. Fe^{2+} and MDA levels were lower while GSH was higher in the 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups compared to Control. Both SJC-treated groups showed higher Fe^{2+} and MDA but lower GSH compared to the 4NQO group, with dose-dependent effects ($P<0.05$). NCOA4 expression was lower while GPX4 expression was higher in the 4NQO group compared to all other groups. Both SJC-treated groups exhibited higher GPX4 expression than Control ($P<0.05$).

Conclusion: Salvia chinensis can interfere with esophageal cancer development, and its mechanism may be related to NCOA4-mediated ferritinophagy.

Keywords: Esophageal carcinoma; Ferroptosis; Salvia chinensis; Carcinoma in situ induction model; Glutathione peroxidase 4; Nuclear receptor coactivator 4

Introduction

Esophageal carcinoma is a malignant tumor of the digestive tract with global impact. According to 2020 statistics, esophageal cancer ranks 8th in overall cancer incidence and 6th in mortality worldwide [1]. The disease often remains occult, with most patients diagnosed at advanced stages, resulting in poor treatment outcomes and prognosis. Its prevalence continues to rise annually due to dietary and emotional factors [2]. Current clinical treatments primarily include surgery, chemotherapy, and molecular targeted therapy, all of which carry significant adverse effects.

Traditional Chinese medicine (TCM) treatment offers the advantages of enhanced efficacy with reduced side effects and has been widely applied clinically. Salvia chinensis, the whole herb of *Salvia chinensis* Benth., is acrid, bitter, and slightly cold in nature. It is used to clear damp-heat, activate blood circulation, and remove blood stasis for treating various diseases including dysphagia, phlegm dyspnea, carbuncles, and scrofula. As documented in *Lei Zheng Zai Zai*, "For dysphagia due to qi stagnation, regulate qi pathways, and Salvia chinensis should be used." Recent pharmacological studies have confirmed the significant anticancer effects of Salvia chinensis and its extracts against various malignant tumors [3], though the specific mechanisms require further investigation.

Ferroptosis is a form of programmed cell death characterized by iron-dependent accumulation of lipid reactive oxygen species [4]. Accumulating evidence indicates that ferroptosis affects numerous cancer targets and may provide novel therapeutic strategies [5-6]. Therefore, this study aimed to investigate the effects and mechanisms of Salvia chinensis intervention in 4-nitroquinoline 1-oxide (4NQO)-induced orthotopic esophageal cancer in C57BL/6 mice, providing a basis for its clinical application.

1. Materials and Methods

1.1 Experimental Animals Ninety SPF-grade female C57BL/6 mice weighing 14-17 g and aged 35-41 days were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All mice were housed in the animal facility of the College of Integrated Chinese and Western Medicine, Hebei Medical University, under a 12-hour light/dark cycle for one week of acclimatization prior to experiments. All experimental procedures were conducted in accordance with the guidelines of the Animal Ethics Committee of The Fourth Hospital of Hebei Medical University (Approval No.: IACUC-4th Hos Hebmu-2022003).

1.2 Drugs and Reagents Salvia chinensis (SJC) granules (Jiangyin Pharmaceutical, batch No. 19092701), 4NQO (Sigma, cat. No. N8141), malondialdehyde (MDA) assay kit, reduced glutathione (GSH) assay kit, and tissue iron assay kit (Nanjing Jiancheng, cat. Nos. A003-1-1, A006-1-1, A039-2-1), glutathione peroxidase 4 (GPX4) antibody (Proteintech, cat. No. 67763-1-Ig), and nuclear receptor coactivator 4 (NCOA4) antibody (Abcam, cat. No. ab86707) were used in this study.

1.3 Experimental Instruments The study utilized the following equipment: 4°C/-20°C refrigerator (Sanyo, Japan), -80°C ultra-low temperature freezer (Sanyo, Japan), TD6001 digital balance (Sigma, USA), BX63 upright microscope (Olympus, Japan), dehydration machine, embedding machine, freezing platform (Wuhan Junjie), pathological microtome (Leica, Shanghai), tissue flattening machine (Jinhua Kedi), and microwave oven (Galanz).

1.4 Experimental Methods 1.4.1 Carcinogen Preparation: Following the established 4NQO-induced mouse orthotopic esophageal cancer model protocol [7], the carcinogen 4NQO was dissolved in 1,2-propanediol to prepare a 2% stock solution, which was stored at -20°C and diluted with purified water to a working concentration of 0.1 g/L before use.

1.4.2 Grouping and Modeling: After one week of acclimatization, mice were randomly divided into four groups: control group (Control, n=15), 4NQO-induced cancer group (4NQO, n=25), low-dose SJC group [4NQO/SJC (91 mg), n=25], and high-dose SJC group [4NQO/SJC (182 mg), n=25]. All groups except Control received 0.1 g/L 4NQO solution ad libitum for 16 weeks, after which they were switched to regular drinking water. All mice received standard chow, while the SJC-treated groups were fed chow mixed with Salvia chinensis granules from week 8 through week 32. The daily doses were calculated based on the clinical dosage of 30-60 g for a 60 kg human, using a conversion factor of 9.1:1 for a 20 g mouse [8], resulting in 91 mg for the low-dose group and 182 mg for the high-dose group.

1.4.3 General Condition and Body Weight Assessment: Mouse activity, mental status, and food and water intake were observed and recorded. Body weight was measured and recorded every 8 weeks.

1.4.4 Tissue Processing: At 32 weeks, mice were euthanized with an overdose of 0.3% pentobarbital sodium (0.05 mL/g). The esophagus was dissected and examined macroscopically. Esophageal tissues were flattened and fixed on an anti-static rubber board to assess surface smoothness, thickening, irregularities, or nodules. After photography, tissues were fixed, dehydrated, cleared, embedded, and sectioned at 4 m thickness.

1.4.5 Hematoxylin-Eosin (HE) Staining and Pathological Analysis: After deparaffinization and hydration, sections were stained with hematoxylin and eosin, dehydrated, cleared, and mounted with neutral balsam. Pathological

changes were observed under a light microscope and evaluated independently by two pathologists; a third pathologist adjudicated any disagreements. Pathological categories included normal epithelium, mild dysplasia, severe dysplasia, and squamous cell carcinoma.

1.4.6 Measurement of Fe^{2+}, GSH, and MDA Content: A portion of esophageal tissue was accurately weighed and homogenized (weight:volume = 1:9) in 0.9% sodium chloride solution. After centrifugation for 10 minutes (radius 8.5 cm, 2,500 r/min), the supernatant was collected for analysis. Absorbance was measured at 520 nm for Fe^{2+}, 532 nm for MDA, and 405 nm for GSH according to the kit instructions, and concentrations were calculated using the provided formulas.

1.4.7 Western Blot Detection of NCOA4 and GPX4 Protein Expression: Esophageal tissue proteins were extracted from each group, and protein concentrations were determined using a BCA kit. Samples were subjected to electrophoresis, transfer, blocking, and incubation with primary and fluorescent secondary antibodies. Protein bands were visualized using an Odyssey detection system. β-actin served as the internal reference, and relative expression levels of NCOA4 and GPX4 were calculated using Image J software.

1.5 Statistical Analysis Data were analyzed using SPSS 27.0 statistical software. Normally distributed continuous data are presented as mean ± standard deviation ($\bar{x} \pm s$). Comparisons among multiple groups were performed using one-way ANOVA, with pairwise comparisons conducted using LSD-t test. Ranked data were analyzed using the rank-sum test. Survival curves were generated using the Kaplan-Meier method and compared using the Breslow test. $P<0.05$ was considered statistically significant.

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2. Results

2.1 Comparison of General Condition, Body Weight, and Survival Rate During the first 8 weeks, no significant differences were observed among groups. By week 16, mice in the 4NQO-treated groups showed reduced activity and listlessness compared to the Control group. In contrast, mice in both SJC-treated groups exhibited greater activity and better mental status than the 4NQO group.

Body weight comparisons revealed no significant differences at week 0 among the four groups ($P>0.05$). However, significant differences emerged at weeks 8, 16, 24, and 32 ($P<0.05$). Post-hoc comparisons showed that body weight in the 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups was lower than Control at all time points. At week 32, both SJC-treated groups had significantly higher body weight than the 4NQO group ($P<0.05$) .

Breslow test results demonstrated statistically significant differences in survival

curves among the four groups (^{2}=9.907, P=0.019). Survival rates were 100% in the Control group, 56% in the 4NQO group, 76% in the 4NQO/SJC (91 mg) group, and 80% in the 4NQO/SJC (182 mg) group [Figure 1: see original paper].

2.2 Gross Morphological Observations The Control group exhibited smooth esophageal walls with good elasticity. The 4NQO group showed marked esophageal wall thickening with poor elasticity and numerous nodules of varying sizes, most commonly in the middle and lower esophagus. Both SJC-treated groups displayed intermediate changes, with smoother esophageal surfaces and fewer nodules compared to the 4NQO group [Figure 2: see original paper].

2.3 HE Staining and Pathological Analysis HE staining revealed no pathological abnormalities in the Control group, with intact basal layers, appropriate epithelial thickness, and orderly cell arrangement. The 4NQO group exhibited abnormal epithelial proliferation, disordered cell arrangement, and pathological changes including keratin pearls. Both SJC-treated groups showed significant improvement in pathological changes, with the high-dose group demonstrating less severe lesions than the low-dose group [Figure 3: see original paper].

Pathological grading at 32 weeks showed: the Control group had no significant pathological changes; the 4NQO group (n=14) exhibited mild dysplasia in 14.3% (2/14), moderate dysplasia in 35.7% (5/14), and squamous cell carcinoma in 50.0% (7/14) of specimens; the 4NQO/SJC (91 mg) group (n=19) showed mild dysplasia in 31.6% (6/19), severe dysplasia in 42.1% (8/19), and squamous cell carcinoma in 26.3% (5/19); the 4NQO/SJC (182 mg) group (n=20) demonstrated mild dysplasia in 45.0% (9/20), severe dysplasia in 40.0% (8/20), and squamous cell carcinoma in 15.0% (3/20). These differences were statistically significant (P<0.001).

2.4 Comparison of Fe^{2+}, GSH, and MDA Content in Esophageal Tissues Significant differences were observed among the four groups in Fe^{2+}, GSH, and MDA content (P<0.05). The 4NQO, 4NQO/SJC (91 mg), and 4NQO/SJC (182 mg) groups showed lower Fe^{2+} and MDA but higher GSH compared to Control. Both SJC-treated groups exhibited higher Fe^{2+} and MDA but lower GSH compared to the 4NQO group. The high-dose SJC group showed higher Fe^{2+} and MDA but lower GSH compared to the low-dose group (P<0.05) .

2.5 NCOA4 and GPX4 Protein Expression Levels Significant differences in NCOA4 and GPX4 protein expression were observed among the four groups (P<0.05). The 4NQO group showed lower NCOA4 but higher GPX4 expression compared to all other groups. Both SJC-treated groups exhibited higher GPX4 expression than Control (P<0.05) , [Figure 4: see original paper].

3. Discussion

In traditional Chinese medicine, esophageal cancer is termed "ye-ge" (esophageal obstruction), with various descriptions by physicians throughout history. Salvia chinensis has been used historically for dysphagia due to its heat-clearing, detoxifying, swelling-reducing, and mass-dispersing effects. Modern research demonstrates significant anticancer efficacy of SJC and its extracts against various malignancies including breast and liver cancer [9-11]. Our previous work confirmed that SJC induces autophagy in esophageal cancer cells via the AMPK/ULK1 signaling pathway [12].

In this study, the 4NQO group developed thickened esophagi with irregular surfaces and multiple nodules, with histopathological changes confirming successful establishment of the orthotopic esophageal cancer model. Compared with the 4NQO group, SJC intervention resulted in thinner esophageal walls, fewer nodules, attenuated pathological changes, and significantly improved survival rates, indicating that SJC effectively suppresses the progression of orthotopic esophageal cancer in mice.

Ferroptosis is initiated by the accumulation of free Fe^{2+}, which activates the Fenton reaction to release free radicals, driving lipid peroxidation, disrupting cellular structures, and generating reactive oxygen species that cause cell death [13-15]. As ferroptosis progresses, lipid peroxides decompose into various compounds including MDA. GSH serves as an antioxidant that neutralizes lipid peroxides and protects membrane fluidity, making it a key antioxidant indicator [16]. Therefore, Fe^{2+}, MDA, and GSH represent important markers of ferroptosis. Our findings demonstrated that SJC intervention significantly increased Fe^{2+} and MDA while decreasing GSH in mouse esophageal tissues compared to the 4NQO group, suggesting that SJC promotes ferroptosis in esophageal cancer by facilitating iron accumulation and reducing antioxidant capacity.

As a novel cell death mechanism, ferroptosis is closely associated with multiple cellular metabolic regulatory systems involving iron metabolism dysfunction, amino acid antioxidant imbalance, and lipid peroxide accumulation [17]. NCOA4 is a key regulator of ferroptosis that primarily functions by mediating ferritinophagy—the degradation of ferritin that releases free iron and leads to iron overload-induced cell death [18]. GPX4 cooperates with GSH to exert antioxidant effects, blocking reactive oxygen species generation and inhibiting ferroptosis in tumor cells [19]. We selected NCOA4 and GPX4 as reference indicators and found that SJC intervention increased NCOA4 protein expression while decreasing GPX4 expression in mouse esophageal tissues compared to the 4NQO group, indicating that SJC promotes ferroptosis in esophageal tumor cells through mechanisms potentially involving NCOA4 and GPX4.

The pathogenesis of esophageal cancer remains incompletely understood. Traditional Chinese medicines, with their multi-component and multi-target char-

acteristics, play significant roles in prevention and treatment but also present challenges in elucidating specific mechanisms. This study provides theoretical support for the use of Salvia chinensis in esophageal cancer treatment and offers new insights into its mechanism of action.

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Note: Figure translations are in progress. See original paper for figures.