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HuangJinShuangShen granules can improve immunity and enhance the ability of 5-fluorouracil to induce apoptosis of gastric cancer cells

Xuefei Xu[1,2,3], Chuqi Yang[1,2,3], Mengting Wu[1,2,3], Qingwen An[1,2,3], Tao Jiang[1,2,3] and Guangji Zhang[1,2,3]*

[1]School of Basic Medical Sciences, Zhejiang Chinese Medical University, Hangzhou, China
[2]Key Labortary of Blood-Stasis-Toxin Syndrome of Zhejiang Province, Hangzhou, China
[3]Traditional Chinese Medicine "Preventing Disease" Wisdom Health Project Research Center of Zhejiang, Hangzhou, China

Abstract: In recent years, Chinese herbal compounds have gained significant prominence in the treatment of gastric cancer. The goal of this study was to investigate the antitumor effect of HuangJinShuangShen granules (HJSS) combined with 5-fluorouracil on MFC gastric cancer mice. In this study, the MFC model with gastric cancer was successfully established. After continuous administration for 14 d, the body weight, tumor volume and weight, and spleen mass of mice in each group were recorded. The levels of IFN-γ and TGF-β_1 in serum were detected by ELISA. The expression of apoptosis proteins in tumor tissues was detected by Western blotting. Compared with the model group and the 5-FU group, the combined drug group can significantly inhibit tumor growth, reduce tumor volume, promote tumor cell necrosis and increase spleen index in mice. At the same time, the combined treatment group significantly increased IFN-γ level and BAX protein expression, decreased TGF-β_1 level and decreased Bcl2, Caspase-9 and Cleaved Caspase-3 protein expressions. These findings provide evidence that HJSS can augment the suppressive impact of 5-FU on tumor growth in gastric cancer mice, potentially through the induction of tumor cell apoptosis and the restoration of immune function.

Keywords: Gastric cancer, Huang Jin Shuang Shen granules, 5-fluorouracil, cell apoptosis, immunity.

INTRODUCTION

Gastric cancer (GC) stands as one of the most prevalent malignancies. In 2020, China recorded 479,000 new cases of gastric cancer, of which 90% were categorized as advanced gastric cancer, leading to 374,000 fatalities (Sung *et al*., 2021). At present, the treatment of gastric cancer in modern medicine mainly focuses on surgical resection, perioperative chemotherapy, and molecular targeted therapy (Joshi and Badgwell, 2021). One commonly employed antimetabolite chemotherapy agent is 5-fluorouracil (5-FU), which serves as a frontline treatment for various solid tumors and as adjuvant chemotherapy. However, its clinical utility often encounters limitations, including drug resistance, a short half-life, and non-specific cytotoxic effects. At the same time, it is prone to bone marrow suppression, cardiotoxicity, gastrointestinal reactions and neurological symptoms (Maeda *et al*., 2018).

In traditional Chinese medicine (TCM), the concept of holism and syndrome differentiation is upheld, which can strengthen the body resistance to eliminate pathogenic factors, stabilize the cancer focus, deter recurrence and metastasis and mitigate the adverse effects of chemotherapy. Consequently, it contributes to enhancing the long-term survival rates and overall quality of life for cancer patients. Therefore, more and more cancer patients choose the integrative medicine to treat cancer, with TCM playing a prominent role in contemporary clinical practice (Ye *et al*., 2023). For example, Pan *et al*. have proved that the addition of Chinese medicine compound during chemotherapy could significantly enhance the survival and overall well-being of patients diagnosed with GC (Pan *et al*., 2020); Liu *et al*. have demonstrated that the co-administration of Compound Kushen Injection with chemotherapy could potentially offer a more effective and less toxic strategy compared to using a single chemotherapy drug for the treatment of triple-negative breast cancer at the single-cell level (Liu *et al*., 2022); Zhu *et al*. revealed that the combination therapy involving Kangai injection and platinum-based chemotherapy has the potential to significantly enhance clinical efficacy, boost immune function and mitigate side effects in the management of advanced non-small cell lung cancer (Zhu *et al*., 2022). HuangJinShuangShen granules (HJSS) was the self-made prescription of our team for GC due to stasis and toxin stagnation syndrome. The prescription was composed of *Astragalus membranaceus*, *Lonicera japonica Thunb.*, *Actinidia arguta* (Sieb. & Zucc) Planch. ex Miq, *Salvia miltiorrhiza Bge.*, *Angelica sinensis* (Oliv.) Diels, *Scrophularia ningpoensis Hemsl.*, and *Glycyrrhiza uralensis Fisch*. In this experiment, the subcutaneous xenografts of 615 mice with MFC gastric cancer cells were used as the research object. The HJSS and 5-FU were combined to study the therapeutic effect of the combination of the two drugs on GC *in vivo*, which provided a new concept for the treatment of GC with utilizing traditional Chinese medicine.

**Corresponding author:* e-mail: zgjtcm@zcmu.edu.cn

MATERIALS AND METHODS

Chemical reagent

Astragalus membranaceus, *Lonicera japonica Thunb.*, *Actinidia arguta* (Sieb. & Zucc) Planch. ex Miq, *Salvia miltiorrhiza Bge.*, *Angelica sinensis* (Oliv.) Diels, *Scrophularia ningpoensis Hemsl.* and *Glycyrrhiza uralensis Fisch.* of HJSS were all purchased from Zhejiang Chinese Medical University Yinpian Co., Ltd. Fluorouracil injection (H50020128) was purchased from Southwest Pharmaceutical Co., Ltd. RIPA lysis buffer (P0013B), PMSF (ST506), SDS-PAGE protein loading buffer (P0015L) and high-sensitivity ECL chemiluminescence kit (P0018S) were purchased from Shanghai Biyun Biotechnology Co., Ltd. BAX antibody (2772), Cleaved Caspase-3 antibody (9664) and Caspase-9 antibody (9508) were purchased from Cell Signaling Technology (CST), the USA. Bcl2 antibody (ab182858) was purchased from abcam, the UK. Mouse-derived GAPDH antibody (830030) was purchased from Aibixin (Shanghai) Biotechnology Co., Ltd. Pre-dyed protein marker (26616) was purchased from Thermo Fisher Scientific Shiyer Technology (China) Co., Ltd. Electrophoresis solution (AR1146-10), transfer solution (AR1151-10) and TBS-T rinse buffer (AR0195-10) were purchased from Wuhan Boster Biological Technology Co., Ltd. HRP-labeled goat anti-rabbit IgG antibody (AS014), HRP-labeled goat anti-mouse IgG antibody (AS003), interferon-γ (IFN-γ) ELISA kit (RK00019) and transforming growth factor-β_1 (TGF-β_1) ELISA kit (RK00057) were purchased from Wuhan ABclonal Biotechnology Co., Ltd.

Preparation of decoction of HJSS

30g of *Astragalus membranaceus*, 15g of *Lonicera japonica Thunb.*, 15g of *Actinidia arguta* (Sieb. & Zucc) Planch. ex Miq, 15g of *Salvia miltiorrhiza Bge.*, 10g of *Angelica sinensis* (Oliv.) Diels, 12g of *Scrophularia ningpoensis Hemsl.* and 6g of *Glycyrrhiza uralensis Fisch.*; decocting the above medicinal materials for 2 times, mixing the decoctions, heating, concentrating to obtain a suspension with a crude drug content of 2.06 g/mL, and storing at 4°C for subsequent use. After comparative analysis of the UPLC from 3 batches of HJSS, we confirmed that the composition of HJSS is stable and can be used for our experiments (fig. S1, table. S1, 2).

Experimental animals and design

Thirty male 615 inbred mice (SPF grade, weighing 20±2 g, and 4–5 weeks) were originated from the Laboratory Animal Center of Institute of Hematology, China Academy of Medical Sciences and raised in the SPF room of the Animal Experimental Center of Zhejiang Chinese Medicine University at 12h/12 h, with automatic light and dark switching (8:30-20:30). The temperature is controlled at (25±2)°C; The relative humidity is controlled at 50 to 70 percent. All animal experiments were followed the regulations of the Zhejiang Chinese Medicine University on the management and use of experimental animals, which all conformed to the 3R principle.

MFC was purchased from Wuhan Punosei Life Technology Co., Ltd. The cells were cultivated in RPMI Medium 1640 medium containing 10% fetal bovine serum and 1% double antibody in a 5% CO_2 incubator at 37°C.

After the establishment of the tumor-bearing mouse model for gastric cancer, they were randomly allocated into 6 groups, each containing six mice. Model group was given normal saline intragastric administration, once a day. The 5-FU group was intraperitoneally injected with 5-FU (20 mg/kg), once/2 days. Combined low-dose, medium-dose and high-dose groups were intragastric administration of HJSS (10 g/kg, 20 g/kg, 40 g/kg), once a day, and intraperitoneal injection of 5-Fu (20 mg/kg), once/2 days, respectively. All groups were treated for 14 consecutive days. During the drug administration, the body weight, mental state, hair, action and other general indications of the mice were observed.

Detecting tumor growth volume

Starting from the initial day of drug administration, we measured the longitudinal (a) and transverse (b) dimensions of subcutaneous tumors using a vernier caliper every 2 days and subsequently calculated the tumor volume.

Tumor volume = $(a \times b^2)/2$

Spleen index determination

24 hours after the last dose, the mice were humanely euthanized via cervical dislocation and the spleen was harvested to determine its mass and the organ coefficient was calculated.

Organ coefficient = (organ mass/body mass) ×10.

ELISA assay

The supernatant of tumor tissue homogenate was prepared. Strictly following the instructions of the kit, the light absorption values of IFN-γ and TGF-β_1 in tumor tissue were measured with ELISA kits and the standard curve was established. The contents of IFN-γ and TGF-β_1 in tissue were calculated.

Hematoxylin-eosin (H&E) staining

The taken fresh tumor tissue specimen was washed with normal saline, fixed by soaking in 4% paraformaldehyde solution, dehydrated, transparentized and waxed conventionally, embedded in paraffin and cut into sections with a thickness of 3.5μm. After HE staining, the sections were sealed with neutral gum and cut into sections. The morphology of tumor cells was observed under a 20-fold optical microscope and photographed for preservation.

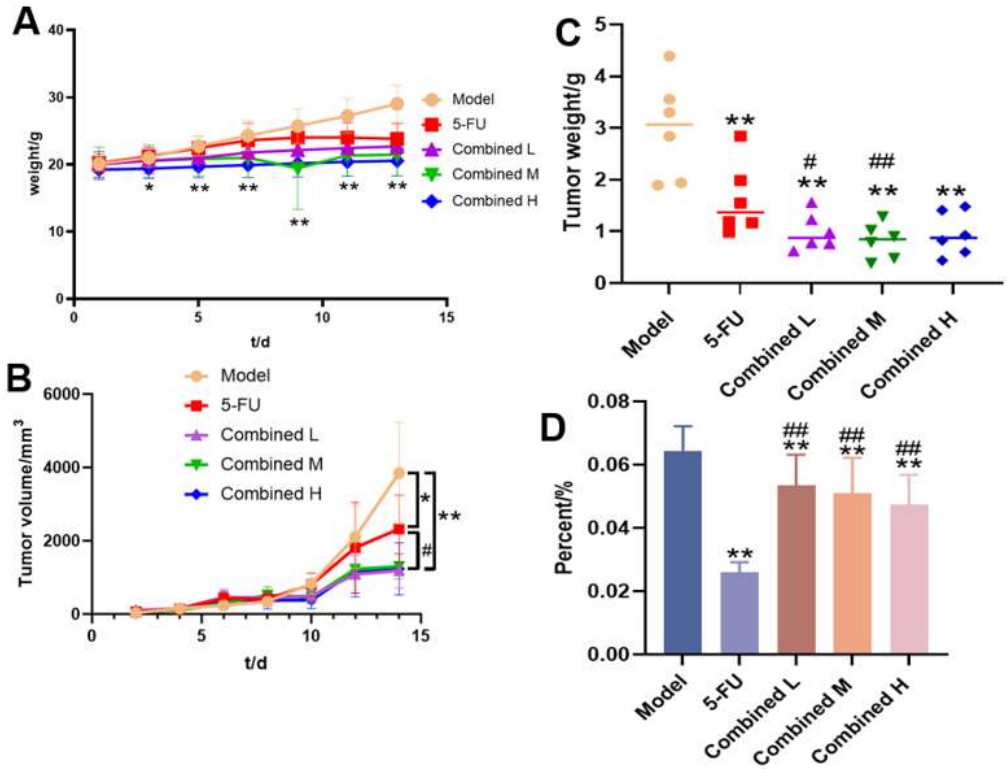

Fig. 1: HJSS reduced the toxic and adverse effects of 5-FU while augmenting its efficacy in inhibiting the proliferation of tumor cells. (A) Body weights, measured every 2 days. (B, C) The weight (B) and volume (C) of xenografts for each dose group. (D) Spleen index of each administration group. Data are expressed as mean ± SD (n = 6); $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 5-FU group.

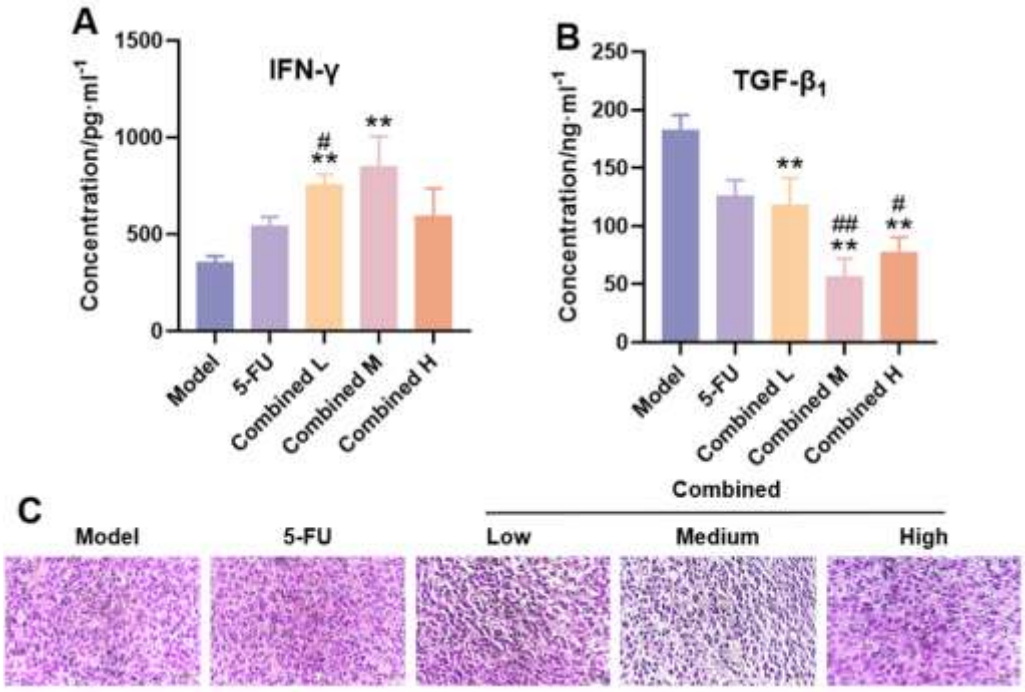

Fig. 2: HJSS regulates the immunity of tumor-bearing mice and enhances the killing effect of 5-FU on tumor cells. (A, B) The results of detecting IFN-γ (A) and TGF-β_1 (B) in tumor tissues using ELISA. (C) Microscopic image of H&E staining results of tumor tissue (HE, ×20). Data are expressed as mean ± SD (n = 3), $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 5-FU group.

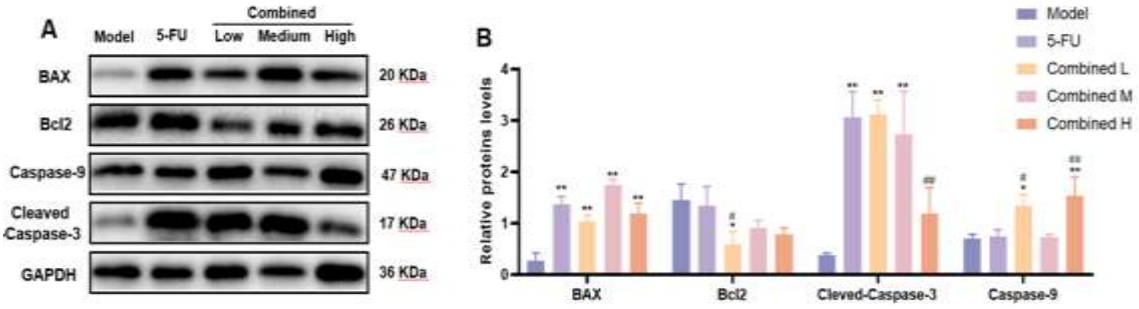

Fig. 3: HJSS enhances the ability of 5-FU to induce apoptosis of gastric cancer cells. (A) The protein levels of BAX, Bcl2, Caspase-9 and Cleaved Caspase-3 were determined by western blot. (B) Grayscale statistics of protein expression relative to internal control. Data are expressed as mean ± SD (n = 3), $^{*}P<0.05$, $^{**}P<0.01$ vs model group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs 5-FU group.

Western blot analysis

The total cellular protein was extracted using a standard method, and its concentration was measured using an ultra-micro spectrophotometer. The protein was denatured and separated by SDS-PAGE, PVDF membrane was transferred and blocked by 5% defatted milk powder. The primary antibody was incubated overnight at 4°C. The secondary antibodies were incubated for 2 h at room temperature. Two reagents A and B in the ECL luminescence kit were mixed in equal volumes in a centrifuge tube and the ECL mixed solution was drop wise added on a PVDF membrane for reaction in the dark for 3-5 mins and developed in a dark room of a gel imaging system. Adjust the exposure time until the best band appears. The intensity of each target band was analyzed using Image J software.

Ethical approval

Committee of Laboratory Animals of Zhejiang Chinese Medical University Laboratory Animal Research Center accepted this study protocol (Approval No. IACUC-202205-01).

STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism 9.0 software. Continuous data were assessed for normal distribution and presented as mean standard ± deviation (x± s). Independent sample t-test was used for comparison between two groups, and one-way analysis of variance was used for comparison among multiple groups. At least three independent experiments were performed. A p-value of less than 0.05 was considered statistically significant.

RESULTS

HJSS can enhance the inhibitory effect of 5-FU on the growth of gastric cancer

As shown in fig. 1A, no significant changes were observed in the body weight of mice among the different administration groups. The tumor volume and mass in each administration group was significantly smaller compared to the model group after two weeks of dosing, and the inhibition effect of the combination of two drugs is better (fig. 1B, C).

HJSS can alleviate the effect of 5-FU on the spleen index of tumor-bearing mice

As shown in fig. 1D, compared with the model, the spleen index of mice in each administration group was significantly reduced. It is worth noting that the spleen index of the combined drug group is significantly higher than that of the 5-FU group.

Effect of HJSS Combined with 5-FU on inflammatory factors of tumor tissue in tumor-bearing mice

As shown in fig. 2A, B, there was a significant increase in IFN-γ levels in mice treated with 5-FU and the combination of low and medium doses and TGF-β_1 levels were markedly reduced. Furthermore, the combination of low and medium doses exhibited a notable increase in IFN-γ levels and a tendency toward decreased TGF-β_1 levels compared with the 5-FU group.

HJSS and 5-FU had a synergistic effect on inflammatory factors in tumor tissue of tumour-bearing mice

As shown in fig. 2C, in the tumor tissue of mice within the model group, the number of tumor cells was abundant, and they were arranged disorderly and densely. The tumor cells showed solid, patchy and diffuse distribution, cytoplasmic eosinophilia, large and hyperchromatic nuclei and basically no necrotic foci. In the 5-FU group, there was a modest reduction in the quantity of tumor cells within the tumor tissues, accompanied by a few necrotic foci. Conversely, in the combination administration group, the number of tumor cells significantly decreased, with sparsely arranged cells exhibiting marked signs of apoptosis, tissue structure damage and a substantial presence of necrotic cells. Particularly, the effect was significant in the combination medium-dose administration group.

Effect of HJSS combined with 5-FU on expression of apoptotic proteins in tumor tissue of mice

As shown in fig. 3, compared with the model group, there was a significant upregulation in the expression levels of

BAX, Caspase-9 and Cleaved Caspase-3 proteins within the tumor tissue, while the expression level of Bcl2 protein was significantly decreased. Furthermore, the combined drug administration group demonstrated a significantly superior therapeutic effect compared to the 5-FU group.

DISCUSSION

Gastric cancer is responsible for an increasing number of cancer-related deaths worldwide. Chemotherapy, a common treatment approach for gastric cancer, not only targets cancer cells but also impacts normal cells, leading to toxicity and apoptosis induction. While 5-FU serves as a frontline drug for gastric cancer treatment, clinical cases often encounter the challenge of 5-FU resistance. This resistance can manifest through various mechanisms and pathways, including alterations in drug transport, evasion of apoptosis, regulation of autophagy, engagement of cancer stem cells, interactions within the tumor microenvironment, epigenetic modifications and disturbances in redox balance (Azwar *et al*., 2021). In recent years, 5-FU has been increasingly utilized in combination with various modulators to treat a wide range of cancers, with a focus on targeting apoptosis and other critical cancer signaling pathways (Ouyang *et al*., 2022). The HJSS possesses the ability to clear heat and detoxify, enhance blood circulation, and alleviate blood stasis. Prior investigations have indicated that certain components within HJSS exhibit positive effects in the treatment of gastric cancer (Kim *et al*., 2019, Ni *et al*., 2022, Wang *et al*., 2017). However, whether the HJSS exerts a better effect on the inhibition of the proliferation of stomach cancer cells is not clear. The outcomes of this research indicated that the combination group exhibited a superior anti-tumor effect compared to the 5-FU group in isolation, which might be due to the fact that HJSS could increase the efficacy and decrease the toxicity of 5-FU, promote the apoptosis of tumor cells and enhance the immunoregulatory ability.

IFN-γ and TGF-β play a key role in the occurrence and development of gastric cancer (Tu *et al*., 2011, Guan *et al*., 2015). IFN-γ is a multi-functional cytokine with antiviral, antitumor, and immune regulatory functions, which is crucial for normal immune homeostasis and plays a key role in tumor monitoring (Sastre *et al*., 2000). Modern pharmacological studies have shown that the active ingredients in HJSS, such as angelica polysaccharide can increase the expression of IFN-γ, thereby enhancing the immune killing ability *in vivo* and achieving the eradication of tumor cells (Liu *et al*., 2019). The TGF-β family has attracted extensive attention due to its many functions in cells and development as well as its role in many diseases, including cancer (Derynck and Budi, 2019). TGF-β_1 is one of the TGF-β subtypes and the main subtype in the tumor microenvironment. In the pre-malignant and early malignant lesions, TGF-β_1 plays an anti-tumor activity. However, in the established tumor models, TGF-β_1 exerts the tumorigenic effect by supporting epithelial-mesenchymal transformation to inhibit the proliferation, differentiation and antitumor functions of various immune cells (de Streel and Lucas, 2021). The results indicated a notable decrease in IFN-γ levels within the model group, alongside a substantial rise in TGF-β_1 levels, but it recovered after drug administration and the effect of the combination group was the best. We hypothesized that the increased IFN-γ after administration may exert antitumor and immunomodulatory effects by inducing Th1 cell differentiation, cytotoxic T lymphocytes, and dendritic cell activation (Mendoza *et al*., 2019). IFN-γ may also activate immune-related cells through the JAK/STAT pathway and PI3K/AKT to maintain the balanced function of the body (Shao *et al*., 2019). It has been proved that TGF-β in tumor cells can also induce epithelial-mesenchymal transformation through PI3K/Akt/mTOR pathway, enhance the invasive and metastatic potential of tumor cells, and promote tumor progress (Baek *et al*., 2017). TGF-β_1 has been shown to induce VEGF expression in endothelial cells and fibroblasts, while VEGF promotes angiogenesis around GC cells, which in turn promotes GC metastasis (Kariya *et al*., 2018, Ma *et al*., 2021). We speculate that decreasing TGF-β_1 levels after administration inhibits the development of gastric cancer by inhibiting epithelial-mesenchymal transformation. These results prove that HJSS combined with 5-FU can exert the synergistic anti-tumor effect by immunoregulating and inhibiting epithelial-mesenchymal transition, and the mechanism may be related to PI3K/AKT signaling pathway.

Apoptosis, also known as programmed cell death, is a tightly regulated physiological process that ultimately results in cell death. It plays a pivotal role in the development of cells and the preservation of tissue homeostasis in animals. Under the degenerative conditions, the damaged apoptotic system is likely to cause cancer, autoimmune diseases, etc. Prior research has demonstrated that the induction of apoptosis is a significant factor in inhibiting cancer cells. Furthermore, many clinical anticancer drugs achieve their therapeutic objectives by triggering apoptosis in tumor cells (Pistritto *et al*., 2016). However, cancer cells can escape from apoptosis by participating in a variety of mechanisms, leading to their uncontrolled proliferation(Jan and Chaudhry, 2019). At present, there are three major apoptosis-related signaling pathways: the mitochondrial apoptosis pathway, the cell death receptor pathway and the endoplasmic reticulum stress pathway (Cohen, 1993, Jeong and Seol, 2008, Wang *et al*., 1999). The mitochondrial apoptotic pathway is a classical intrinsic apoptotic pathway (Thongsom *et al*., 2017) and Bcl2 family proteins and cysteine protease (Caspase) are the

key proteins for regulating and executing this pathway (Sastre *et al*., 2000). Therefore, protein expressions of BAX, Bcl2, Caspase-9 and Cleaved caspase-3 were detected in the present study. The results illustrated that the combination of HJSS with 5-FU activated the Caspase cascade, inducing apoptosis in gastric cancer cells through the mitochondrial apoptosis pathway, thus exerting the antitumor effects. Research data have found that PI3K/AKT signaling pathway is highly correlated with the occurrence and development of gastric cancer (Fattahi *et al*., 2020). PI3K/AKT pathway is highly activated in gastric cancer cells, which causes a series of malignant proliferation of cells and the cell apoptosis is inhibited, the cell cycle process is accelerated and cells will undergo invasion and metastasis. The PI3K/Akt signaling pathway, which inhibits programmed cell death and regulates multiple proteins or families related to apoptosis, such as inhibitor of apoptosis proteins, Bcl2 family and Caspase-9, is a key anti-apoptotic signaling pathway (Lv *et al*., 2017). Therefore, we speculated that the activation of mitochondrial apoptotic pathway in gastric cancer cells by the combination of HJSS and 5-FU might be related to the inhibition of PI3K/Akt signaling pathway. However, in this study, the effect of the combination of HJSS and 5-FU on the expression of PI3K/Akt signaling pathway-related proteins has not been further studied, which is the shortcoming of this study and also the exploration needed in our subsequent experiments.

CONCLUSION

In conclusion, HJSS can enhance the tumor inhibitory effect of 5-FU *in vivo*, including reducing tumor volume, reducing tumor weight, and inducing tumor tissue apoptosis. At the same time, HJSS may also regulate the level of inflammatory factors in the tumor micro environment and restore the anti-tumor immune function. This study provides experimental basis for the clinical application of HJSS in tumor adjuvant therapy, and also provides a certain scientific reference for the future clinical treatment of malignant tumors.

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