

Original Research Article

The traditional Chinese medicine (TCM), Sheng-xue-xiao-ban, inhibits the angiogenesis in ovarian cancer in vitro

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Abstract

Purpose: The traditional Chinese medicine (TCM), Sheng-xue-xiao-ban (SXXB) is reported to be effective in treatment of thrombocytopenic purpura and chemotherapy-induced thrombocytopenia in clinical practices. The purpose of this study was to explore the functions of the SXXB Capsule in ovarian cancer in vitro.

Methods: The toxicity of the medicine was measured using CCK8 method in ovarian cancer cell lines A2780 and OC-3-VGH and also the human ovarian epithelial cell line IOSE80. The cells were treated using the DMSO-diluted SXXB solution with the concentration levels at 0, 3.125 and 6.25 µg/ml. Western blot was applied to measure the VEGFA proteins in each group. Later, the cells treated with 0 and 25 µg/ml SXXB solution for 48h were collected for supernatant. Then HUVECs were co-cultured with the supernatant. Migration and tube formation assays were performed thereafter.

Results: SXXB solution showed higher toxicity in ovarian cancer cells than in IOSE80. The protein levels of VEGFA were reduced as the SXXB concentrations increased in ovarian cancer cells but not in IOSE80. Migration and formed tubes were inhibited in HUVECs co-cultured with the supernatant collected from SXXB-treated ovarian cancer cells.

Conclusion: The SXXB Capsule might inhibit the angiogenesis in ovarian cancer in vitro.

Keywords: Angiogenesis; VEGFA; ovarian cancer; TCM

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INTRODUCTION

In all gynecologic cancers, high mortality rate was observed in ovarian cancer[1]. The

prognosis for patients diagnosed at advanced stage is poor[1, 2]. Recently, the anti-angiogenesis therapies have been an alternative for patients with ovarian cancer, which might improve the prognosis [3].

The traditional medicine Sheng-xue-xiao-ban capsule (SXXB) has been widely reported to be effective in treatment of thrombocytopenic purpura [4] and chemotherapy-induced thrombocytopenia in clinical practice. The major ingredients of the SXXB are indigo naturalis, cortex moutan, forsythia, hairyvein agrimony and licorice [5], among which, indigo naturalis contains tryptanthrin, which showed its anti-angiogenesis function as previously reported [6-8]. Psoriasis vulgaris, featuring abnormally increased angiogenesis, epidermal proliferation, keratinization, etc. [9], could be alleviated by topical application of indigo naturalis ointment. Due to its anti-inflammation, anti-bacteria, anti-virus, immunomodulatory functions, indigo naturalis was discovered to exert positive effect in colitis [10, 11], and leukemia [12]. Cortex moutan contains β -sitosterol, gallic acid, paeonol and quercetin [13, 14] and it has shown the anti-inflammation, anti-oxidative and anti-cancer effects as previously reported [14-17]. To note, paeonol could induce apoptosis and autophagy in epithelial ovarian cancer cells [18] and paeonol was also reported for its anti-angiogenesis effect previously [19], which enables cortex moutan to possess anti-angiogenesis property [20]. Licorice contains bioactive compounds, glycyrrhetic acid, which could inhibit angiogenesis and isoliquiritigen, which could suppress ovarian cancer metastasis [21]. Although the ingredients of SXXB contain the bioactive components that could inhibit angiogenesis, there has been no study revealing whether SXXB could regulate angiogenesis *in vitro*, nor was there a study exploring whether SXXB could regulate ovarian cancer *in vitro*. Therefore, in this study, we first evaluated the inhibitory rates of SXXB solution in ovarian cancer cells and normal ovarian epithelial cells. Thereafter, VEGFA protein change was examined in ovarian cancer cells treated with SXXB. Finally, HUVECs incubated with tumor-conditioned media after different SXXB treatment were used for migration and tube formation assays [22]. This study was to reveal the unknown effect of SXXB in ovarian cancer in relation to angiogenesis.

METHODS

The preparation of SXXB solution

Following approved by the Ethical Committee of Tianjin Medical University Cancer Institute and Hospital, China SXXB Capsules (0.45g) were ground with 563 μ l DMSO. Then PBS was used for dilution and 0.22 μ m filter was for filtering.

Then the storage concentration of SXXB was 80mg/ml.

Cell culture

The human ovarian cancer cell lines A2780 and OC-3-VGH, and the human normal ovarian epithelial cell line IOSE80 were all cultured in RPMI-1640 medium with 10% FBS (Evergreen, Zhejiang, China). The human umbilical vein endothelial cells (HUVECs) were cultured in DMEM and 10% FBS. Cells were all purchased from Procell (Wuhan, China).

CCK8 method

Different concentrations of SXXB were achieved using PBS. The concentrations, 0, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μ g/ml were finally selected for toxicity test. Cells were treated with the indicated concentrations of SXXB solution for 24, 48h in 96-well plates. Then the CCK8 kit (Beyotime, Nantong, China) was applied for the toxicity test in each cell line. OD values were read on a microplate reader at 450nm. The experiments were conducted in triplicate. The inhibition rate was calculated based on the equation: Inhibition rate = $1 - \frac{\text{mean of OD values of the group}}{\text{mean of OD values of the untreated group}}$.

Western blot

The cells were treated with 0, 3.125 and 6.25 μ g/ml SXXB solution for 48h. The RIPA lysis buffer for western blot (Bioss, Beijing, China) was used for protein extraction. Then standard procedure was followed as described previously [23]. The antibodies used in this study included, the anti-VEGFA (1:500, 20393R, Bioss), anti-GAPDH (1:5000, 52262R, Bioss) and the Goat anti-Rabbit antibody (1:5000, 40295G-HRP, Bioss). The relative protein expression was analyzed on Image J (NIH, USA).

The treatment of HUVECs

The cell lines including A2780, OC-3-VGH, IOSE80 were treated with 0 and 6.25 μ g/ml SXXB solution for 48h and then the supernatant was collected respectively. Then the HUVECs were cultured with addition of the supernatant collected as indicated for 24h. Then the HUVECs were collected for the migration and tube formation experiments.

Migration

Transwell method was applied to examine the migration of HUVECs after the supernatant

treatment. The cell suspension was added in the upper wells without serum while 10% FBS was added in the lower wells. After cultured in cell incubator (Thermo Fisher, Shanghai, China) for 24h at 37°C, the membranes were placed in 70% methanol for 60min and crystal violet solution was used to stain the cells. The membranes were observed under the lab microscope and the cells were counted from 3 different fields.

Tube formation assay

Growth factor reduced Matrigel was placed in refrigerator at 4°C for thawing (BD Biosciences, CA, US). After Matrigel was polymerized, the HUVECs after supernatant treatment, were suspended in RPMI-1640 medium. Then the cells (30000 cells per well) were seeded on the 24-well plates coated with Matrigel. The Nikon TS2 microscope (Nikon, Japan) was used to take the images at 100X. The number of tubes was counted under the microscope.

Statistical analysis

Graphpad Prism 9.0 was employed to analyze the statistical data in this study. Kruskal-Wallis test was used to evaluate the significance of differences among groups. $P < 0.05$ was considered as significant.

RESULTS

SXXB solution induced more significant cytotoxicity in ovarian cancer cells

After cells were treated with SXXB solution for 24, 48 and 72h, CCK8 experiments were carried out to measure the cytotoxicity. In cells treated with SXXB for 24h, the viability was not influenced by the increase of SXXB concentration (Figure 1A-C). However, after SXXB treatment for 48h, the ovarian cancer cells including A2780 and OC-3-VGH presented significant inhibition while the IOSE80 cells didn't (Figure 1D). The inhibition rates were demonstrated in Figure 1E and listed in Table 1. The results showed that in general, the ovarian cancer cell lines A2780 and OC-3-VGH were actively responsive to the increasing concentrations of SXXB treatment (Figure 1E). When cells were treated with concentrations lower than 50µg/ml, the inhibition rates ranged from 3 to 16.38% in A2780 cell group and from 9.5 to 20.43% in OC-3-VGH while the inhibition rates ranged 1.41 to 4.69% in IOSE80 cell group (Table 1).

Table 1: Inhibition rates in cells after 48h treatment of SXXB solution

Concentration (ug/ml)	IOSE80 (%)	A2780 (%)	OC-3-VGH (%)
0	0	0	0
3.125	4.28	3	9.5
6.25	1.41	6.98	13.44
12.5	2.21	10.13	17.38
25	4.69	16.28	20.43
50	6.09	26.56	23.3
100	10.71	34.66	27.87
200	21.89	38.48	39.52
400	28.65	49.29	46.51
800	32.4	55.51	53.32

SXXB solution could inhibit the VEGFA protein in ovarian cancer in vitro

The cells were pretreated with 0, 3.125 and 6.25µg/ml SXXB solution for 48h. Then the cells were collected for western blot analysis. Results showed that VEGFA protein expression was decreased in the ovarian cancer cells after the treatment of 3.125 and 6.25µg/ml but no significant changes were observed in the IOSE80 cell line (Figure 2), suggesting that SXXB might impact on the angiogenesis in ovarian cancer in vitro.

The migration and tube formation of HUVECs could be inhibited in tumor-conditioned cells with SXXB treatment

The cells were treated with SXXB (0 and 6.25µg/ml) for 48h and then the supernatant was collected. The HUVECs cultured with the supernatant for 24h were collected for further migration and tube formation experiments. The results showed that HUVECs migration was suppressed in the tumor-conditioned groups with SXXB treatment but not in the IOSE80-conditioned groups (Figure 3). Tube formation results also showed that tube formation was significantly reduced in tumor-conditioned groups with SXXB involvement but not in the IOSE80-conditioned groups (Figure 4).

DISCUSSION

Angiogenesis is a process that new blood vessels sprout out of the existing ones[24]. In solid tumors, microvessels provide nutrients and oxygen to maintain the growth and metastasis of the solid tumors [25-27]. Therefore, angiogenesis is closely engaged in tumor growth and metastasis in ovarian cancer [26]. Recent years have witnessed the emerging of targeted therapies including anti-angiogenesis therapy[28]. In most cases, the anti-angiogenesis is to target VEGFA [29]. For instance, bevacizumab, a monoclonal antibody of vascular endothelial growth factor A(VEGFA), is recommended to apply in combination

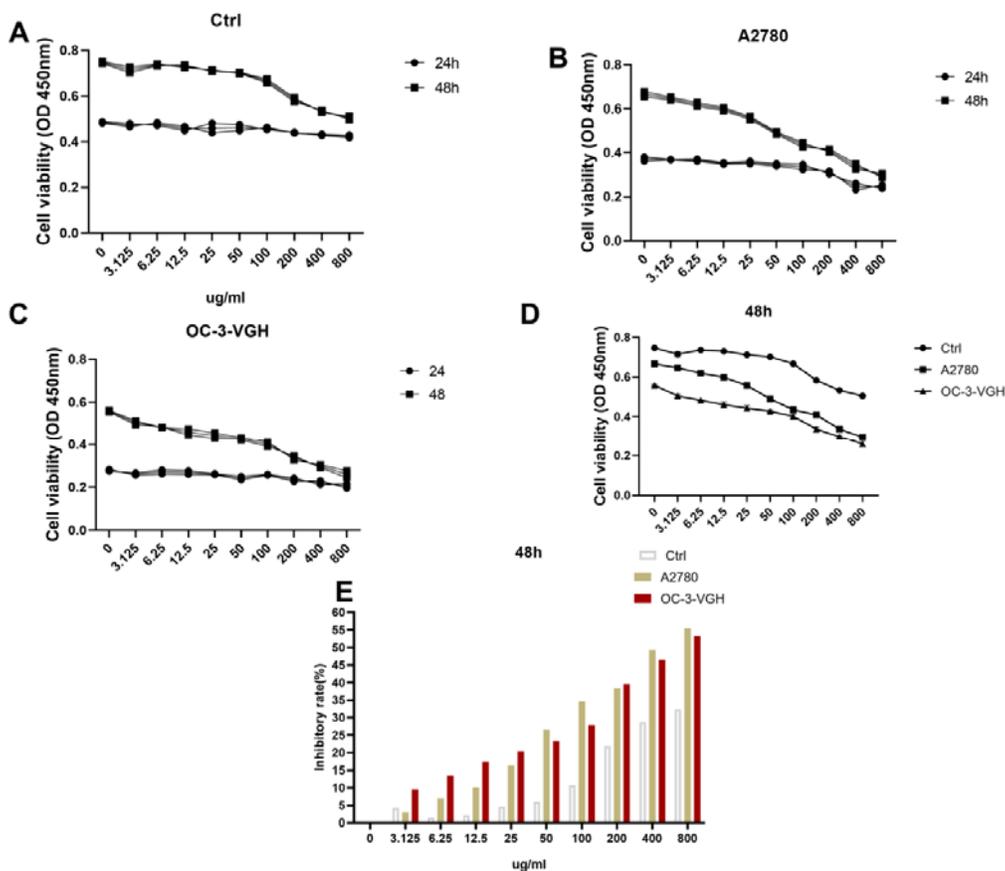


Figure 1: SXXB solution induced more significant cytotoxicity in ovarian cancer cells. Cells were treated with SXXB solution (0, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 $\mu\text{g/ml}$) for 24 and 48h. CCK8 methods were applied to analyze the cell viability (A-D) and inhibition rates (E).

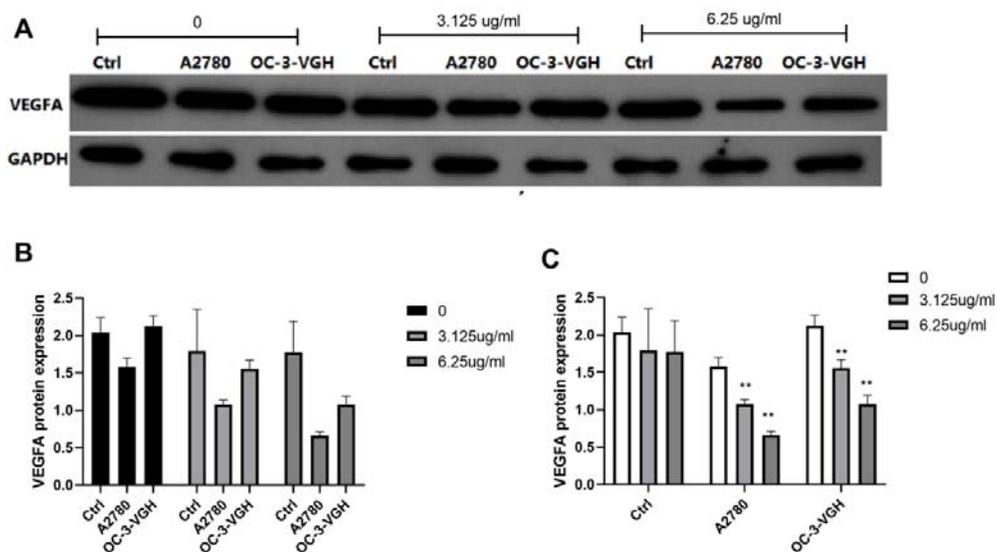


Figure 2: SXXB solution could inhibit the VEGFA protein in ovarian cancer in vitro. The cells were pretreated with 0, 3.125 and 6.25 $\mu\text{g/ml}$ SXXB solution for 48h. Then the cells were collected for western blot analysis. Image J was used to analyze the relative expression of VEGFA normalized to GAPDH. Kruskal-Wallis test was applied. $**P < 0.05$.

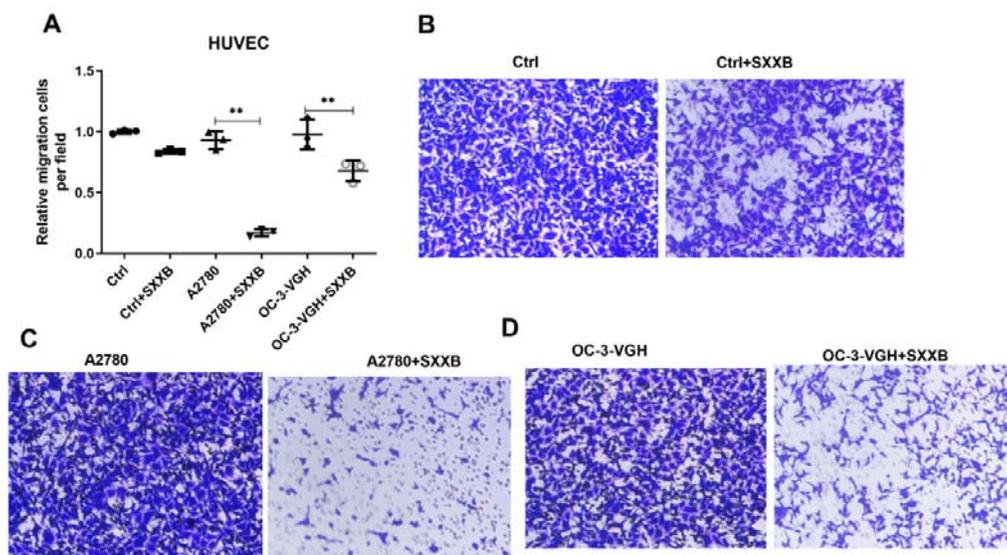


Figure 3: The migration of HUVECs could be inhibited in tumor-conditioned cells with SXXB treatment. The cells were treated with SXXB (0 and 6.25 μ g/ml) for 48h and then the supernatant was collected. The HUVECs cultured with the supernatant for 24h were collected. Transwell method for migration was then performed and the cells that migrated were counted under microscope from 3 different fields(A-D). Kruskal-Wallis test was applied. $**P < 0.05$

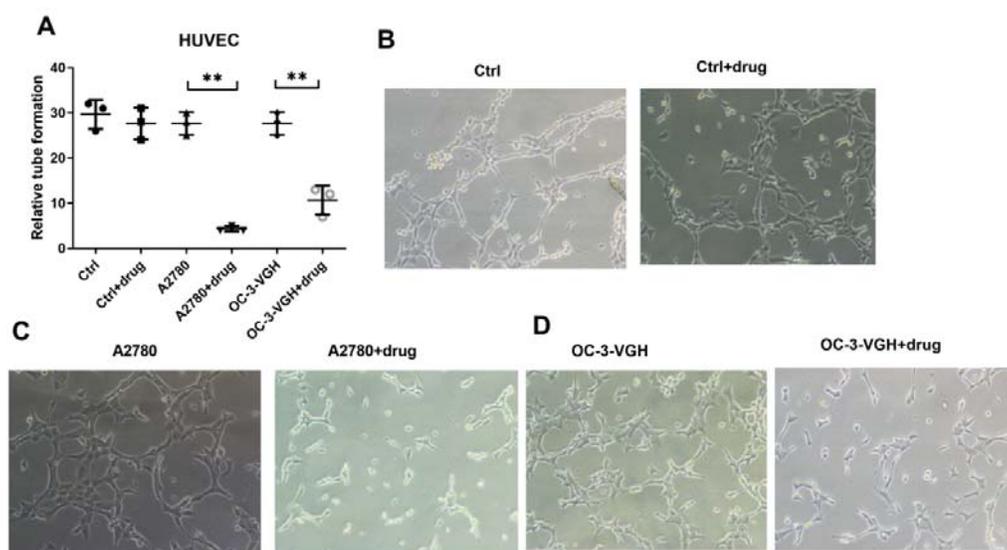


Figure 4: The tube formation of HUVECs could be inhibited in tumor-conditioned cells with SXXB treatment. The cells were treated with SXXB (0 and 6.25 μ g/ml) for 48h and then the supernatant was collected. The HUVECs cultured with the supernatant for 24h were collected. Tube formation was performed and the number of tubes were counted under microscope (A-D). Kruskal-Wallis test was applied. $**P < 0.05$

with chemotherapy for patients with ovarian cancer [30, 31]. Particularly for those patients diagnosed at advanced stage, anti-angiogenesis therapy remains an option [32].

In vitro, angiogenesis is manifested by tube formation, migration and VEGFA protein expression [33]. Therefore, in this study, we first examined the cytotoxicity induced by SXXB solution in ovarian cancer cells A2780 and OC-3-VGH and normal

epithelial cell IOSE80, which reveals that the viability of ovarian cancer cells was significantly inhibited by the SXXB (with concentration lower than 50 μ g/ml). Then VEGFA protein expression was inhibited in ovarian cancer cells after SXXB treatment. Furthermore, the HUVECs incubated with the supernatant from the tumor cells after SXXB treatment were examined for migration and tube formation, suggesting that the SXXB might inhibit the angiogenesis in ovarian cancer in vitro. Tumor cells

are able to secrete the pro-angiogenesis factors, leading to the formation of abnormal blood vessels [34]. In this study, the SXXB could regulate the cancer cell viability and might modulate the secretions from the cancer cells, contributing to the inhibition in angiogenesis in vitro. However, the tumor microenvironment is complex, comprising a variety of cell types like immune cells, endothelial cells, epithelial cells, fibroblasts and also the extracellular components around tumor cells and nourished by the vascular system [35-37]. The extracellular components include growth factors, cytokines, extracellular matrix, hormones, etc [38, 39]. Therefore, although this study confirmed that SXXB could mediate the angiogenesis in ovarian cancer in vitro, further studies should be performed to explore the potential regulatory mechanisms involved. In addition, xenograft models could also be established to validate whether the SXXB is effective in inhibiting angiogenesis in vivo.

CONCLUSION

In this study, SXXB was revealed to be less responsive to the normal epithelial cells. The inhibition rates were significant in ovarian cancer cells treated with SXXB even at a low concentration level of 12.5µg/ml. Further, we confirmed that SXXB could regulate the angiogenesis in ovarian cancer in vitro as supported by the findings from tube formation and migration assays. However, further studies should be carried out to explore the underlying regulatory mechanisms in vitro and also validate the therapeutic effect of SXXB through angiogenesis inhibition in xenograft models.

DECLARATIONS

Abbreviations

Traditional Chinese medicine (TCM); Sheng-xue-xiao-van capsule (SXXB Capsule)

Ethical approval

The cell and animal experiments were performed by strictly following the regulations of Tianjin Medical University Cancer Institute and Hospital. No human sample was used in this study.

Availability of data and materials

All data are included in this published article and its Additional files.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Enqi Kong designed and carried out the experiment, Chengshuang Wang and Yingchun Zheng wrote the draft of the article, Haiping Song conducted experiments and collected data, Li Sun and Jing Tian prepare for the survey. All authors read and approved the paper for publication.

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