International Core Journal of Engineering Volume 8 Issue 3, 2022
ISSN: 2414-1895 DOI: 10.6919/ICJE.202203_8(3).0029

Flavonoids from Millettia Speciosa Champ Inhibit the Migration and Angiogenesis of Prostate Cancer Cells

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Abstract

Prostate cancer is one of the most common cancers in men. At present, many treatment and prevention strategies are used to treat prostate cancer, but the poor prognosis of patients is still a major problem. Niu Dali(Millettia speciosa Champ)is a medicinal and food-homologous plant commonly found in South China. The rhizomes of Niu Dali have been clinically proven to have therapeutic effects on many chronic diseases, in which flavonoids and polysaccharides are the main active molecules. In this study, we investigated the effect of flavonoids on prostate cancer cells on migration and angiogenesis in vitro and in vivo. The results indicated that flavonoids from Millettia speciosa Champ (FMSC) were capable of significantly inhibiting the migration ability of mouse prostate cancer cell line RM-1 and human prostate cancer cell line PC-3. Meanwhile, in the C57 mouse subcutaneous prostate cancer model, FMSC could inhibit prostate cancer tumor growth and angiogenesis with the dose-dependent pattern. Taken together, FMSC isolated from Niu Dali plays a role in treating prostate cancer.

Keywords

Prostate Cancer; Millettia Speciosa Champ; Flavonoids.

1. Introduction

Prostate cancer is one of the most common cancers in men. The incidence of prostate cancer increases significantly over 50 in men[1]. According to the data on global cancer in 2020 at the World Health Organization's International Agency for Research on Cancer (IARC), the number of new cases of prostate cancer is 1.41 million, accounting for 7.3% of annual cancer cases and ranking second in the incidence of cancer in men.[2]. Depending on the patient's PSA level, Gleason score, and disease progression, treatment options include androgen castration, prostatectomy, and radiation therapy[3]. However, current drug therapies available to treat this malignancy often cause adverse effects and poor prognosis. It leads to poor quality of life for patients. Therefore, it is necessary to find natural drugs that are non-toxic and have more minor side effects to prevent or treat prostate cancer.

Millettia Speciosa Champ(M.speciosa) is a traditional medicinal plant of the legume family, and its roots are commonly used in China to treat chronic diseases such as cough and rheumatism. Flavonoids and polysaccharides are its main active components[4]. Many studies have shown that flavonoids have anti-tumor, anti-inflammatory, antioxidant, and other pharmacological activities[5, 6]. However, few studies have focused on the anti-tumor effects of FMSC, especially in prostate cancer. The impact of FMSC on prostate cancer is unclear. Tumor metastasis is the spread of cancer cells from the primary tumor site to distant organs and is the most common cause of death in cancer patients. It was involved in the migration and invasion of cancer cells, cell adhesion, and vascular formation[7]. In our study, to study the therapeutic effect of FMSC on prostate cancer cells, we conducted MTT Assay to examine the impact of FMSC on the growth activity of prostate cancer cells in PC3 cells and RM-

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1 cells. We conducted a wound-healing Assay to observe the effects of FMSC on tumor migration. Subcutaneous prostate cancer was established in C57BL/6 mice to study the impact of FMSC on angiogenesis. We found a significant inhibitory effect of FMSC on the growth of the prostate. After treatment with FMSC, PC3 and RM-1 cells were significantly inhibited in the MTT assay. The tumors were smaller in mice by the gavage of FMSC. FMSC was found to remarkably inhibit the migration ability of PC3 and RM-1 cells by wound-healing assay. FMSC can reduce angiogenesis in animal experiments, and a significant decrease in VEGF-A was detected. The same applies to cellular experiments. In addition, a dose-dependent effect of FMSC was observed in our experiments.

2. Materials and Methods

2.1 The Experimental Materials

Millettia Speciosa Champ is provided by the Chinese Academy of Tropical Agricultural Sciences in Danzhou city, Hainan Province. The plants are 5-6 years old. FMSC was isolated and identified by Shixiu Feng in Xianhu Botanical Garden, Shenzhen, China.

2.2 Animal Treatments

The subcutaneous prostate cancer tumors were established in C57BL/6 mice to determine the effect of FMSC on prostate cancer. RM-1 cells were digested, and the cell concentration was adjusted to 2×106 / mL. 2×105 RM-1 cells were injected subcutaneously into the inguinal side of male C57BL/6 mice at 6-8 weeks. Mice were randomly divided into four groups: control group (PBS, 5% ethanol), low-dose group (FMSC,50 mg/kg), medium-dose group (FMSC,100 mg/kg) and high-dose group (FMSC,150 mg/kg). During 21 days of continuous gavage, the mice's body weight and tumor growth were examined. At the end of the experiment, the mice were mercifully killed and dissected. The subcutaneous tumors in the abdomen of the mice were peeled and weighed.

2.3 Cell Culture

The PC3 cells, HUVEC cells, and RM-1 cells were purchased from the American Type Culture Collection (ATCC; USA). The RM-1 cells were cultured in Dulbecco's Modified Eagle's Medium medium (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA). The PC3 and HUVEC cells were cultured in DMEM/F-12(Sigma, USA) containing 10% fetal bovine serum (FBS; Gibco, USA). They were all incubated at 37 °C in a humidified atmosphere of 5% CO2. The medium was changed every two days with a fresh medium.

2.4 Cell Viability Assay

MTT assay was performed to determine cell viability. In general, Cells in good growth condition were digested with trypsin (Gibco, USA). The cells were seeded in 96-well plates at a density of 5000 cells per well. After post-adhesion starvation with serum-free medium for 12 hours, cultures containing different concentrations of FMSC were added for 24 h and 48 h. Fresh culture media containing 0.5 mg/ml of MTT (Solarbio, China) was added to each well at 37 ℃ for 4 h. Dimethyl sulfoxide was added to each well after removing the MTT solution. A multifunctional microplate reader measured the absorbance at OD 490 nm (Biotek, USA).

2.5 Wound-healing Assay

When the cells in the 12-well plate reached 100% confluence, the tip of the 200 μL spear was used to make vertical scratches on the bottom of the cell culture plate, and a serum-free medium containing different concentrations of FMSC was added for culture. Before culture, photos were taken under an inverted microscope, recorded as 0 h, and the scratch area was calculated. Cultured RM-1 cells and PC3 cells were photographed at the exact location of 0h 24 h and 48 h later, denoted as 24 h and 48 h, and the scratch area was calculated.

2.6 HE Staining

Tumor tissue was removed from the mice subcutaneously and cut to the appropriate size. Fix the fresh tissue in 4% paraformaldehyde fixative. Excess water was removed from the tissue surface and

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dehydrated. Tumor tissue was embedded with an OTC embedding agent and stored at -80 °C. Sections were stained with hematoxylin for 10 min and eosin for 2 min at a thickness of 5 μm. After dehydration and transparency treatment, areas were sealed with neutral adhesive and photographed under a microscope.

2.7 Real-time PCR

The effect of FMSC on the expression genes associated with angiogenesis was detected by real-time reverse-transcription polymerase chain reaction(RT-PCR). Total RNA was extracted from cells using TRIZOL (AG, Guangzhou, China). According to the manufacturer's protocol, 500 ng total RNA was transcribed into cDNA using an RT kit (AG, Guangzhou, China). Quantitative real-time PCR (QRT-PCR) was performed using Power SYBR Green Master Mix (AG, Guangzhou, China) and a real-time PCR detection system (Bio-RAD). Primers were synthesized by Sangon Biotech Company (Shanghai, China). All the reactions were run in triplicate. Primers used for RT-PCR are listed in Table 1.

Table 1. Primer Sequences for Real-Time PCR.

Numble	Primers	Forward	Reverse
1	VEGF-A	TAGAGTACATCTTCAAGCCGTC	CTTTCTTTGGTCTGCATTCACA
2	β-actin	GGCACCACACCTTCTACAATGAG	AGAGGCATACAGGGACAGCAC

2.8 Western Blot Analysis

The effect of FMSC on the expression of angiogenesis-related proteins was examined by Western blotting. Cells or tumor tissues were lysed using RIPA lysate for 30 min. The protein samples were obtained by centrifugation at 12,000 rpm for 10 min at 4°C. The total protein concentration was determined by the BCA kit(BCA Protein Assay Kit, Bioss, China), and the concentration of protein samples was adjusted to 5 ng/μl. Distinct proteins of different molecular weights by SDS-page electrophoresis, and then transfer the proteins on the gel to the PVDF membrane(pore size: 0.45 μm, Millipore, USA). The membranes were blocked with 5% skim milk powder for 2 hours. After Washing with TBST 3 times, the membranes were incubated with anti-VEGF-A (1:1000; Cell Signaling Technology, USA) and anti-β-actin (1:1000; Cell Signaling Technology, USA) at 4 °C overnight. Secondary antibodies(1:2000, Cell Signaling Technology, USA) were incubated for 1 hour at room temperature. Fluorescence intensity was observed under a chemiluminescence instrument with ECL luminescent solution, and protein expression levels were obtained by gray analysis.

2.9 Statistical Analysis

All data are expressed as mean ± standard deviation. A one-way ANOVA was utilized between groups of results for statistical analysis. The student test compared two groups using GraphPad Prism 7.0 software. The value of $p < 0.05$ was considered significantly different.

3. Results

3.1 Effects of FMSC Treatment on Proliferation of Prostate Cancer Cells

The effect of FMSC on the proliferative capacity of RM-1 cells and PC3 cells was examined by the MTT Assay. As shown in Figure 1, the viability of RM-1 cells was 45.97% and 45.95% after treatment with 400 μg/mL of FMSC for 24 and 48 hours. It demonstrated that 400 μg/mL was the semi-inhibitory concentration IC50 of FMSC on RM-1 cell growth. The maximum inhibitory effect of FMSC was achieved at 600 μg/mL, and the inhibitory effect of FMSC was only slightly enhanced with the extension of FMSC treatment time. There is no significant time-dependent effect observed.

The cell viability of PC3 cells decreased after incubation with different concentrations of FMSC for 24 h. In particular, PC3 cell viability decreased gradually and significantly at 600, 800, and 1000 μg/mL FMSC concentrations. We extended the treatment time to 48h, and we found that the

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inhibitory effect of FMSC on PC3 steadily increased with the increase of FMSC treatment time. After incubation with different concentrations of FMSC for 48 h, the viability of PC3 cells started a decreasing trend at a concentration of FMSC 400 μg/mL. The 1000 μg/mL was the semi-inhibitory concentration IC50 of FMSC on PC-3 cell growth. Compared with the treatment for 24 h, the inhibition of PC3 cells in the 600, 800, and 1000 μg/mL FMSC groups increased by 10.38%, 8.23%, and 16.68%. The experimental results showed that the FMSC treatment significantly inhibited the proliferation ability of PC3 and RM-1 cells, and the inhibitory effect of FMSC on the growth of PC3 and RM-1 gradually increased with the increase of FMSC concentration. It has a specific dose-dependent effect. The FMSC had a temporal effect in PC3 cells, while it was not detected in RM-1 cells.

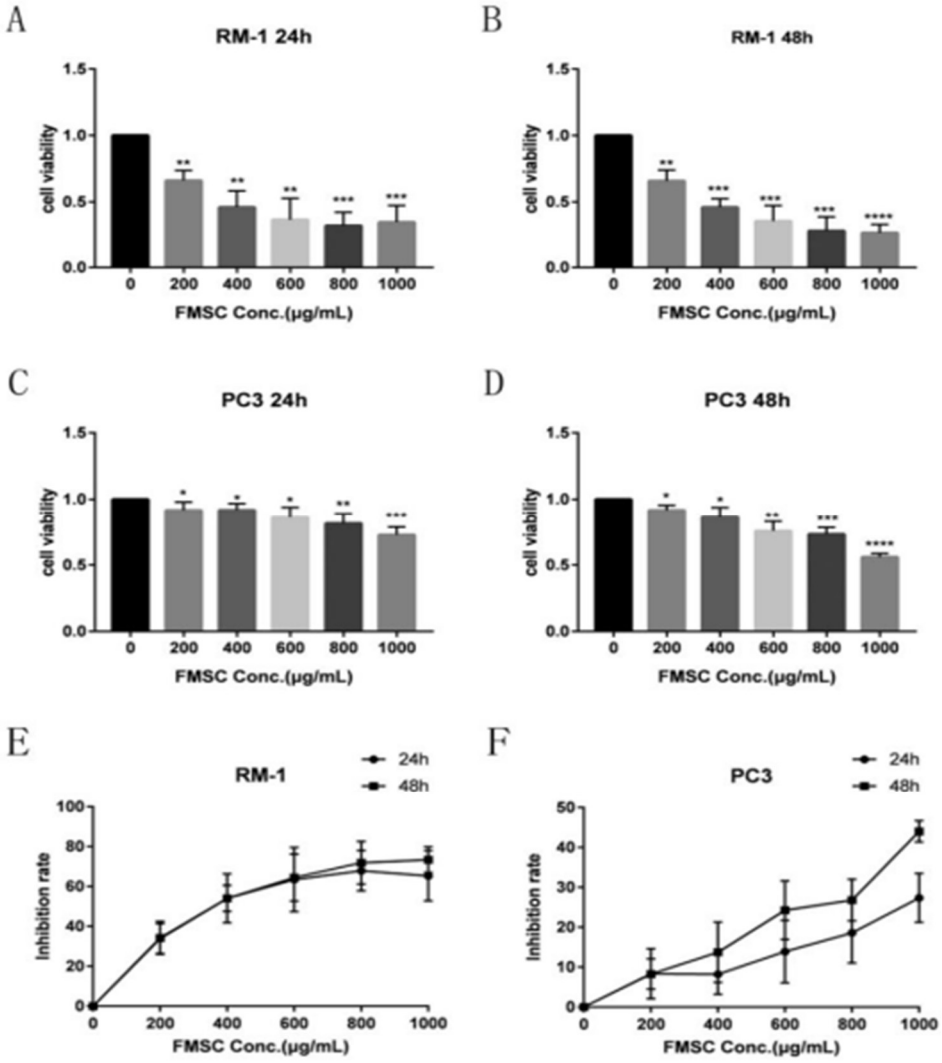

Figure 1. Cell viability and growth inhibition rates of RM-1 and PC3 after treatment with different concentrations of FMSC. RM-1 (A, B) cells and PC3 (C, D) cells were treated with 0, 200, 400, 600, 800, 1000 μg/ mL FMSC for 24h (A, C) and 48h (B, D). Cell activity was detected by the MTT assay and cell growth inhibition rate was calculated (E, F). Values represent the mean ± SD of three independent experiments. compared with control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.2 FMSC Inhibits the Migration of RM-1 and PC3 in Prostate Cancer Cells

Tumor metastasis is the most significant factor in cancer progression leading to death, which involves cell adhesion, migration, invasion, and angiogenesis. Cancer cell migration is critical in forming new

ISSN: 2414-1895 DOI: 10.6919/ICJE.202203_8(3).0029

tumor focus and necessary for tumor vascularization [8]. The migratory movement of cancer cells is a process of cell pseudopod extension and new adhesion establishment in response to migratory and tropism stimuli. The scratch assay was used to examine the effect of FMSC on the migration of PC3 and RM-1 cells. As shown in Figure 2, compared with the scratch distance at 0h, the scratch distance of RM-1 cells in the control group at 24h and 48h was significantly narrower, and there was pseudopod formation on both sides of the scratch. In this experiment, we could find that the migration ability of RM-1 cells was slightly inhibited after treatment with 200 μg/mL FMSC and significantly inhibited at 400 μg/mL FMSC for 24 and 48 h. The migration ability of RM-1 cells was more strongly inhibited at 800 μg/mL FMSC. The scratches were almost unchanged, while the migration rate was significantly reduced by 56.48% and 63.53% at 24 and 48 hours. It demonstrated a dose-dependent effect of FMSC on the migration ability of RM-1, but no time-dependent effect was found.

Similarly, the effect of FMSC on the migratory capacity of PC3 cells was examined using the wound-healing assay. We found that the migratory ability of PC3 cells was more substantial than that of RM-1 cells. The cells in the control group migrated to the middle of the initial scratch after 12 hours, and the scratch was almost completely closed after 24 hours. PC3 cells were treated with different concentrations of FMSC for 12h and 24h. As shown in Figure 3, the migration ability was significantly inhibited with 200 μg/mL FMSC treatment. Its migration rate was inhibited by 45.09% and 58.32% after 12 and 24 h. Half of the inhibition rate had been achieved. However, there was still a tendency to migrate to the middle of the scratch. The migratory ability of PC3 cells treated with 400 μg/mL FMSC was more significantly inhibited. No significant change in the scratch width was observed. Moreover, we almost did not observe any migration of PC3 cells with 800 μg/mL FMSC for 12 and 24 hours. It was found that the migration ability of PC3 cells decreased by 84.36% and 91.39% with 800 μg/mL FMSC for 12 and 24 hours. A substantial inhibitory effect of FMSC on the migratory ability of PC3 could be found. In our study, the FMSC could dose-dependently inhibit the migration ability of PC3 and RM-1 cells, and the inhibitory effect of FMSC on PC3 migration ability was more potent than on the RM-1 cell.

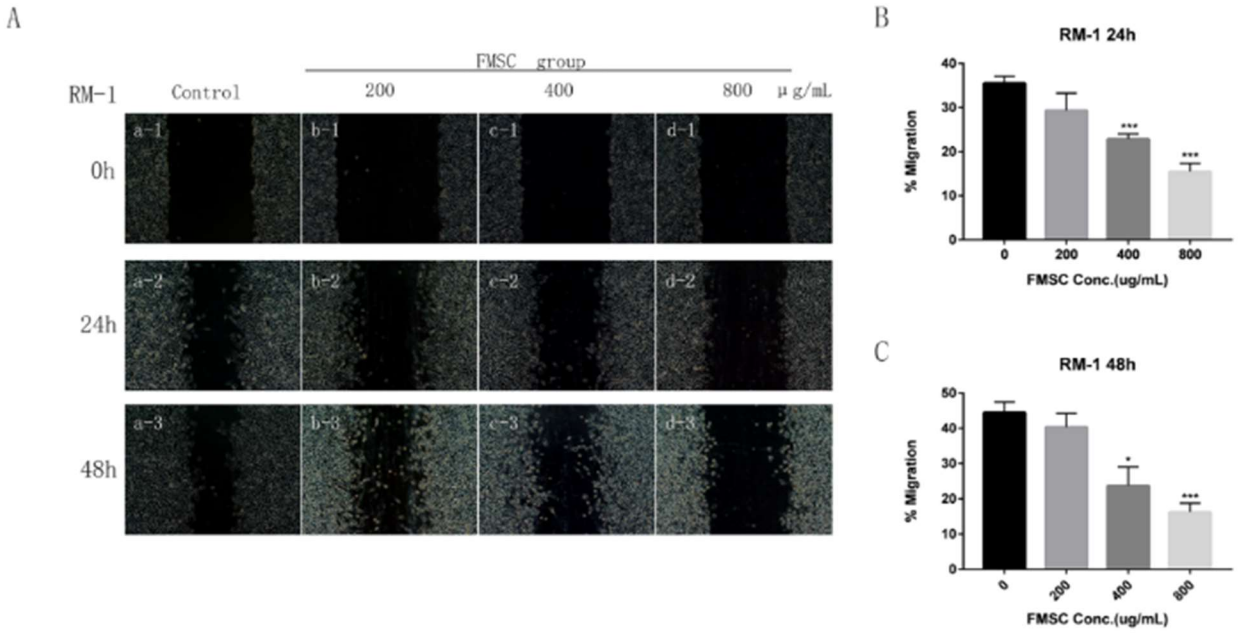

Figure 2. Effect of FMSC treatment on the migratory capacity of RM-1 cells. The migration of RM-1 cells was analyzed by wound-healing assay. Photographs were taken at 0 h, 24 h, and 48 h (A). Wound closure was analyzed for mobility at 24h (B) and 48h (C). Images were taken under a phase-contrast microscope at a magnification of 200x. Values represent the mean ± SD of three independent experiments. Compared to control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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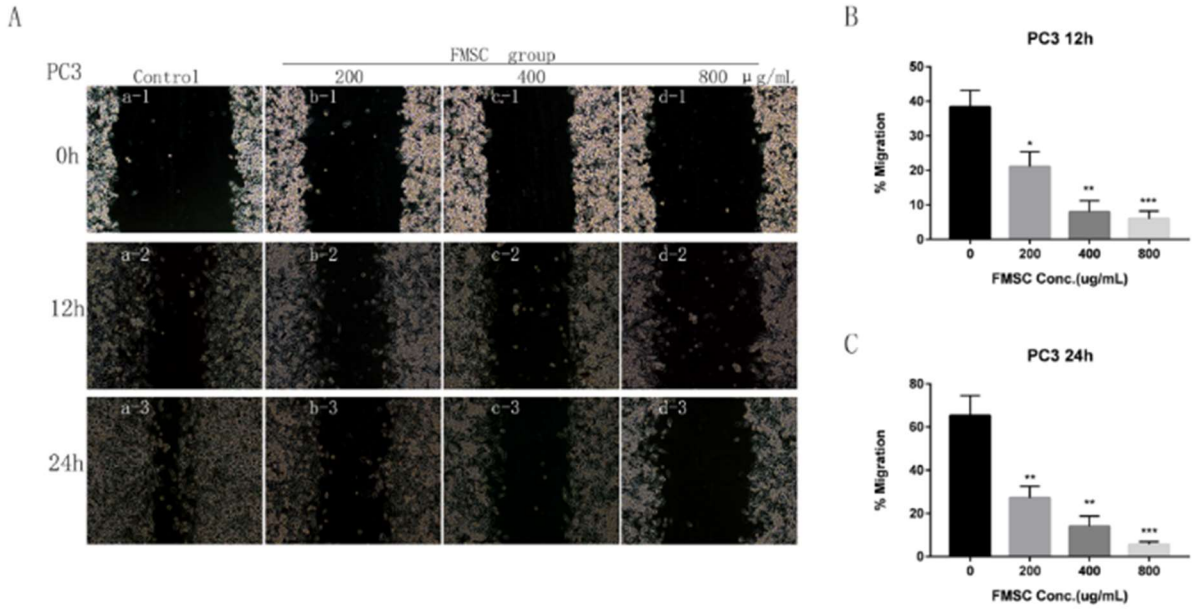

Figure 3. Effect of FMSC treatment on the migratory capacity of PC3 cells. Migration of PC3 cells was analyzed by wound-healing assay. Photographs were taken at 0 h, 24 h, and 48 h (A). Wound closure was analyzed for mobility at 24h (B) and 48h (C). Images were taken under a phase-contrast microscope at a magnification of 200x. Values represent the mean ± SD of three independent experiments. compared to control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.3 Effect of FMSC Treatment on Vascular Distribution in Tumor Tissue

Tumor surface congestion was clearly observed in the control group. In contrast, no apparent blood vessel formation was observed in the FMSC-treated group. We performed HE staining experiments on the tumor tissue sections. The nuclei were stained blue, while the blood vessels were stained red. As shown in Figure 4, thick and long tumor blood vessels were distributed in the tumor tissues of the control group. No evident distribution of blood vessels was found in the FMSC-treated group, and the size of tumor tissues in the 150 mg/kg FMSC-treated group was significantly smaller than the other groups.

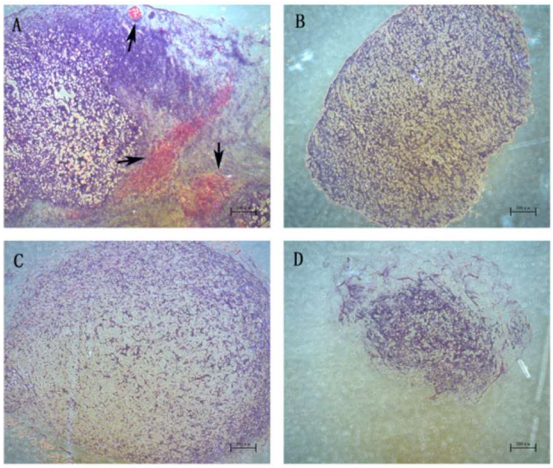

Figure 4. Effect of FMSC treatment on the distribution of blood vessels in tumor tissues. The C57BL/6 tumor-bearing mice were established by subcutaneous transplantation of RM-1 cells. The stripped tumor tissue blocks were subjected to HE staining after being treated with 0 mg/kg, 50 mg/kg, 100 mg/kg, and 150 mg/kg FMSC by gavage for 21 days. A: control; B: 50 mg/kg FMSC ;C: 100 mg/kg FMSC; D: 150 mg/kg FMSC. Arrows point to tumor vessels (red) and blue to nuclei.

ISSN: 2414-1895 DOI: 10.6919/ICJE.202203_8(3).0029

3.4 Effects of FMSC on the Growth of Human Umbilical Vein Endothelial Cells (HUVEC)

The reason why benign tumor cells restrict their growth and metastasis is because of inadequate blood supply. Dormant tumor cells can be activated by the formation of blood vessels. The tumor interacts with the vascular endothelium and secretes growth factors to form the tumor masses [9]. HUVEC are human umbilical cord vein endothelial cells. They are the best model system to study the endothelial cells and their regulatory mechanisms of response to different stimuli. In this experiment, HUVEC cells were initially used to study the effect of FMSC on vascular endothelial cell growth. As shown in Figure 5, after treating HUVEC cells with 100, 200, 400, 600, and 800 μg/mL FMSC for 48 h, 100 μg/mL FMSC had no significant effect on the growth of HUVEC cells and slightly promoted the development of HUVEC cells. The proliferative capacity of HUVEC cells decreased with FMSC concentrations above 200 μg/mL. When the treatment concentration reached 600 μg/mL, the cell viability decreased by 61.73%. It indicates that the semi-inhibitory concentration of FMSC in HUVEC cells ranged from 400 ~ 600 μg/mL. The growth of vascular endothelial cells was significantly inhibited. It was tentatively demonstrated that FMSC could inhibit the remodeling of blood vessels and thus the growth of tumors.

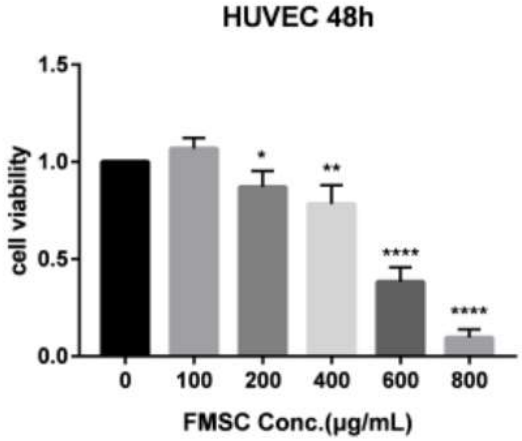

Figure 5. Effect of FMSC treatment on the viability of HUVEC cells. HUVEC cells were treated with 0, 100, 200, 400, 600, 800 μg/mL for 48 h. Cell activity was measured by MTT method. Values represent the mean ± SD of three independent experiments. compared to control,* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

3.5 Effects of FMSC on VEGF-A Expression in Tumor Tissues

Vascular endothelial growth factor (VEGF) is the most prominently induced molecule and plays a predominant role in angiogenesis. To further investigate the effect of FMSC on the expression of angiogenic factors in prostate cancer, we detected the mRNA expression level and protein level of VEGF-A in RM-1 cells and prostate tumors by real-time fluorescence quantitative PCR and Western blot. As shown in Figure 6, VEGF-A mRNA expression was down-regulated in RM-1 cells at 200 and 400 μg/mL FMSC compared with the control group. The down-regulation of VEGF-A mRNA expression was more pronounced at 800 μg/mL FMSC than at 200 and 400 μg/mL. VEGF-A protein was also detected significantly decreased by WB assay in RM-1 cells. VEGF-A protein levels were significantly downregulated by 32.95% at 400 μg/mL FMSC. Similarly, the VEGF-A mRNA and protein expression significantly down-regulated in mouse tumor tissues. The mRNA expression of VEGF-A decreased by 27.11%, and the protein expression decreased by 40.30% at 50 mg/kg FMSC in tumor tissues. VEGF-A mRNA expression and protein levels in tumor tissues were more significantly downregulated at 100 and 150 mg/kg FMSC. The mRNA expression of VEGF-A in tumor tissues was downregulated by 46.75% at 100 mg/kg and 60.89% at 150 mg/kg. The protein expression of VEGF-A was downregulated by 50.20% at 100 mg/kg and 53.14% at 150 mg/kg. Our experiments showed that FMSC treatment significantly inhibited VEGF-A expression in prostate

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cancer cells RM-1 and tumor tissues, and the presentation of VEGF-A also decreased with the increase of FMSC concentration. Thus, FMSC can inhibit angiogenesis in prostate tumors. Our experiments showed that FMSC treatment significantly inhibited VEGF-A expression in RM-1 cells and tumor tissues, and the presentation of VEGF-A decreased with the increase of FMSC concentration. Thus, FMSC would inhibit angiogenesis by suppressing the production of VEGF-A.

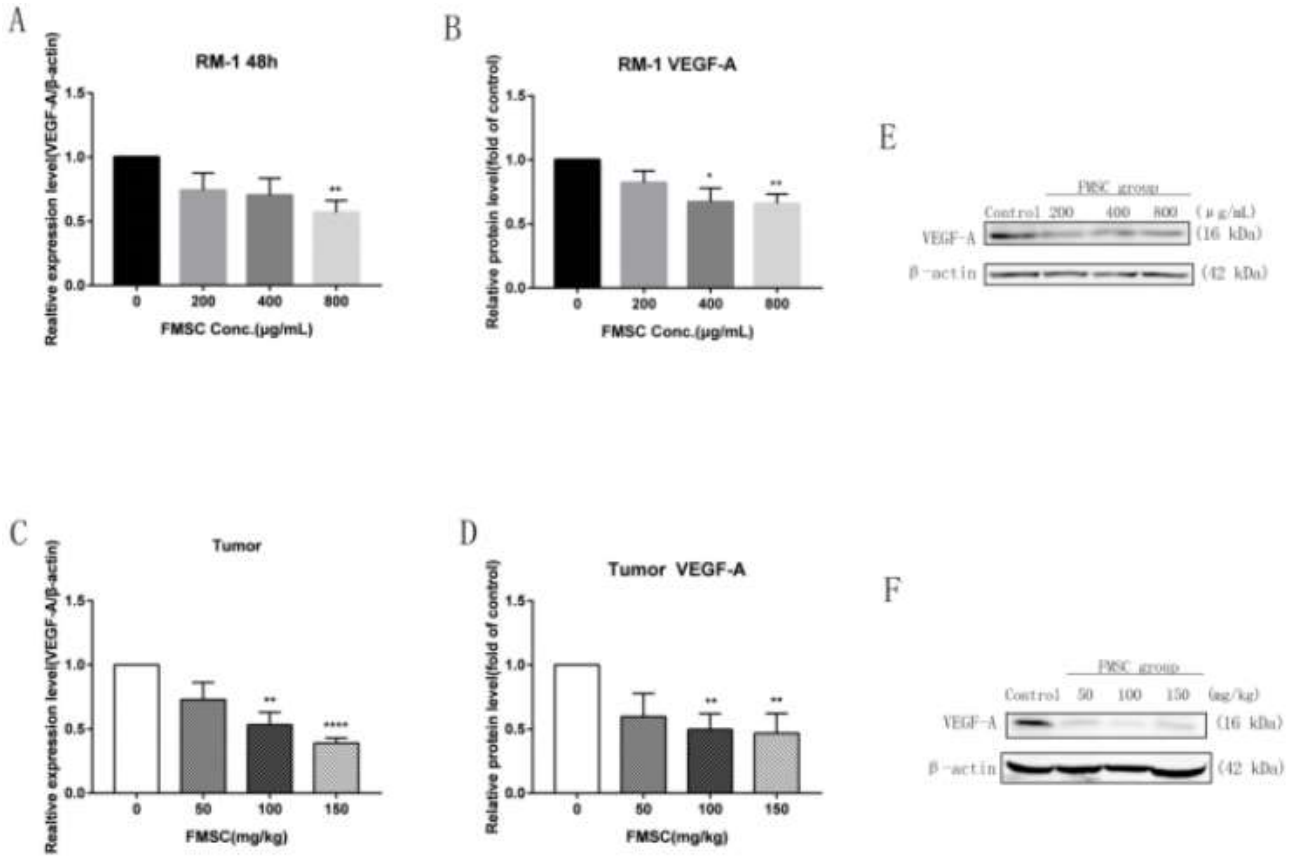

Figure 6. Effect of FMSC on VEGF-A expression in RM-1 cells and tumor tissues. RM-1 cells were treated with 0, 100, 200, 400, 600, 800 μg/ mL for 48 h. The expression of VEGF-A was detected by real-time fluorescence quantitative PCR (A). the protein level of VEGF-A was detected by western blot (B, E). VEGF-A expression in mouse prostate tumor tissues was detected by real-time fluorescence quantitative PCR (C). protein levels of VEGF-A were detected by western blot (D, F). Values represent the mean ± SD of three independent experiments. compared with controls,* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4. Discussion

Millettia speciosa Champ is a traditional medicinal plant of southern China, grows in the deep mountains. The plant is highly adaptable to the natural environment. It is easy to expand and cultivate because of its strong vitality. People cook its roots for consumption to help strengthen their immune systems. Several studies have been conducted to show the superior effectiveness of Millettia speciosa Champ in the treatment of rheumatoid arthritis [10], arthralgia [10], chronic bronchitis, and inflammatory diseases [11]. Not only does Millettia speciosa Champ have significant antioxidant effects [12], but it has also been found to have immunological activity [13] and anti-fatigue effects in mice experiments [14]. In addition, it has been discovered to have antidepressant effects [15]. Millettia speciosa Champ has a complex composition, and many physicochemically active substances have been identified. The main active ingredients include alkaloids, coumarins, polysaccharides [4]. Furthermore, Millettia speciosa Champ is rich in flavonoids, containing more than ten flavonoids such as mangosteen, 2',5',7-trihydroxy-4'-methoxy isoflavone, glycyrrhizin [16, 17]. Forty-eight

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components, including 21 isoflavone substances, were detected using a chemical analysis method based on ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) from Millettia Speciosa Champ root in 2019. [18]. In recent years, more and more studies have shown the role of flavonoids in preventing and treating cancer [19, 20]. As a traditional Chinese medicine rich in flavonoids, few studies explore its role in cancer, especially prostate cancer. In our research, FMSC was found to inhibit prostate cancer's growth, angiogenesis, and migration for the first time.

In our study, we demonstrated in vitro and in vivo that FMSC inhibited the growth of prostate cancer, especially in terms of angiogenesis and migration of cancer cells. In vitro, MTT showed that FMSC significantly inhibited the development of human prostate cancer cell line PC3 and murine prostate cancer cell line RM-1. The IC50 concentration of FMSC on RM-1 cells at 24 and 48 h was around 400 μg/mL, while FMSC also significantly inhibited the growth of PC3 cells, but its IC50 concentration was around 1000 μg/ mL. The inhibitory effects of FMSC on RM-1 cells and PC3 cells were dose-dependent and time-dependent. Meanwhile, the same results were obtained in the in vivo prostate cancer model mice. After treatment with FMSC, the growing tumor masses of allograft cancer cells in C57BL/6 mice were significantly smaller. The reduction of blood vessels in the tumor masses was observed considerably by HE staining of frozen sections. Detecting the expression of VEGF-A in tumor tissues and RM-1 cells, we found that the expression of VEGF-A decreased with the increase of FMSC. Our results suggest that FMSC has an inhibitory effect on angiogenesis in prostate cancer. In addition, We examined the viability response of HUVEC to FMSC and found it also had the same inhibitory effect on human angiogenesis. In addition to the angiogenesis aspect, it also had a significant inhibitory effect on cell migration. In our study, The results of the cell scratching experiment showed that the mobility of PC3 and RM-1 cells was significantly reduced after the addition of FMSC. In the RM-1 cell, concentrations up to 800 μg/mL of FMSC already inhibited half of the migration rate, and the migration rate was dose-dependent. In the PC3 cell, the inhibitory effect of FMSC was even more pronounced. At a concentration of 200 μg/mL, half of the migration rate was inhibited, and the inhibitory effect was also dose-dependent.

In conclusion, the medical effects of FMSC in the treatment of prostate cancer were investigated for the first time in this study. In the present study, FMSC inhibited the growth of prostate cancer by suppressing the angiogenesis of prostate cancer and inhibiting the migration and spread of cancer cells, thus achieving a positive therapeutic effect. However, the specific signaling pathways it affects remain to be explored.

Acknowledgments

The work was supported by grants from the Forestry Science and Technology Innovation Project of Guangdong Province (Project number: 2021KJCX013).

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