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Basic Study

Effect of moxibustion on VEGF and EGF expressions in tumor tissues of rats with gastric tumor

艾灸对胃荷瘤大鼠肿瘤组织中VEGF与EGF表达的影响

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Abstract

Objective: To explore the inhibitory effect of moxibustion on tumor growth and metastasis, and also its possible mechanism, in gastric tumor-bearing rats by investigating the expressions of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF).

Methods: Fifty healthy Sprague-Dawley (SD) rats (half male and half female) were routinely housed for 1 week. A total of 20 rats were randomly divided into a blank group and a sham operation group, with 10 rats in each group. The remaining 30 rats were used to make gastric cancer models by implantation of ascites-type Walker-256 cancer cells. After successful modeling, rats were randomly divided into a model group, a moxibustion group and an infrared group, with 10 rats in each group. From the day of modeling, the body weight of each group was weighed every 4 days. Warm moxibustion was alternately performed at two-group acupoints [Zhongwan (CV 12), Guanyuan (CV 4) and bilateral Zusanli (ST 36) in one group, and bilateral Pishu (BL 20) and Weishu (BL 21) in another group] in the moxibustion group. The body surface projection area of the stomach was irradiated with short-wave infrared rays in the infrared group, once a day, 20 min per time for 21 d. At the end of the treatment, the gastric tumor was completely dissected, and the tumor volume and tumor growth inhibition rate were calculated. Then the gastric tumor cell metastasis was recorded. The levels of VEGF and EGF in rat gastric tumor tissues were determined by enzyme-linked immunosorbent assay (ELISA).

Results: Compared with the blank group, the body weight of the model group decreased significantly after modeling ($P<0.05$); compared with the model group, the rats in the moxibustion group had increased body weight during the middle and late stages (both $P<0.05$). The tumor volumes of rats in the moxibustion group and the infrared group were smaller than the volume in the model group (both $P<0.05$). The tumor growth inhibition rate in the moxibustion group was significantly higher than that in the infrared group ($P<0.05$). The case number of tumor metastasis in the moxibustion group was smaller than that in the model group and the infrared group. The VEGF level in the tumor tissues of the model group was statistically significantly higher than that in the blank group ($P<0.05$). Compared with the model group, the VEGF levels in the moxibustion group and the infrared group were statistically significantly lower (both $P<0.05$). The EGF levels in the tumor tissues of the model group was statistically significantly lower than that in the blank group ($P<0.05$); compared with the model group, the EGF levels in the moxibustion group and the infrared group were statistically significantly increased (both $P<0.05$).

Conclusion: Moxibustion can increase the body weight, inhibit the tumor growth, invasion and metastasis in gastric tumor-bearing rats, which may be related to the regulation of VEGF and EGF expressions in tumor tissues.

Keywords: Acupuncture-moxibustion Therapy; Moxibustion Therapy; Moxa Stick Moxibustion; Neoplasms; Stomach; Vascular Endothelial Growth Factor; Epidermal Growth Factor; Rats

【**摘要**】**目的**：通过观察艾灸对胃荷瘤大鼠肿瘤组织中血管内皮生长因子(VEGF)和表皮细胞生子因子(EGF)表达量的影响，探讨艾灸对胃荷瘤大鼠肿瘤生长、转移的抑制情况及其可能的作用机制。**方法**：将 50 只健康 Sprague-Dawley (SD)大鼠(雌雄各半)常规饲养 1 周后，随机选取 20 只分为空白组和假手术组，每组 10 只。剩余 30 只种植腹水型 Walker-256 癌细胞制作胃癌模型，造模成功后随机分为模型组、艾灸组和红外组，每组 10 只。自造模当日开始，每隔 4 d 称量各组大鼠体质量。艾灸组在两组穴位(中脘、关元和双侧足三里为一组，双侧脾俞和胃俞为一组)上交替施以温和灸，红外组在腹背部胃体表投影区域用短波红外线照射，两组均每日治疗 1 次，每次 20 min，连续治疗 21 d。治疗结束后，完整剥离胃肿瘤，并计算肿瘤体积及肿瘤生长抑制率，记录大鼠胃肿瘤细胞转移情

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况。用酶联免疫吸附法(ELISA)测定大鼠胃肿瘤组织中 VEGF 和 EGF 含量。**结果**：与空白组比较，模型组大鼠造模后体质量明显降低(P＜0.05)；与模型组比较，艾灸组大鼠干预中、后期体质量增加(均 P＜0.05)。艾灸组和红外组瘤体体积均较模型组缩小(均 P＜0.05)；艾灸组肿瘤生长抑制率高于红外组，差异有统计学意义(P＜0.05)；艾灸组肿瘤转移例数少于模型组和红外组。模型组大鼠肿瘤组织中 VEGF 含量明显高于空白组，差异有统计学意义(P＜0.05)；与模型组相比，艾灸组和红外组肿瘤组织中 VEGF 均明显降低，差异有统计学意义(均 P＜0.05)。模型组大鼠肿瘤组织中 EGF 含量较空白组低，差异有统计学意义(P＜0.05)；与模型组比较，艾灸组和红外组肿瘤组织中 EGF 含量均明显升高，差异有统计学意义(均 P＜0.05)。**结论**：艾灸能增加胃荷瘤大鼠体质量，并有抑制胃荷瘤大鼠肿瘤生长及浸润转移的作用，其抑制作用可能与调整胃荷瘤大鼠肿瘤组织中 VEGF 和 EGF 表达量有关。

【关键词】针灸疗法；灸法；艾条灸；肿瘤；胃；血管内皮生长因子；表皮细胞生长因子；大鼠

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Tumor is a neoplasm formed by the abnormal cell proliferation of local tissues due to various tumorigenic factors, and often manifests as a local mass. In China, with the development of social economy, urbanization and the aging of population, the incidence and mortality of malignant tumors continue to increase[1]. Gastric cancer is one of the most common malignant tumors in humans, seriously affecting the quality of human lives. Its poor therapeutic effect is due to delayed treatment or tumor infiltration and metastasis. The invasion and metastasis of gastric cancer is the result of multi-step, multi-stage and multi-factor interaction. The specific mechanism is still unclear[2]. To investigate a way that can effectively control tumor growth and metastasis has become an urgent need for clinical gastric cancer treatment. In this study, we investigated the expressions of vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in the tumor tissues of gastric tumor-bearing rats to explore the effect of moxibustion on tumor growth and metastasis, which was compared with the effect of infrared therapy to further confirm the role of moxibustion in tumor suppression and the mechanism of moxibustion in tumor inhibition.

1 Materials and Methods

1.1 Laboratory animals and grouping

Six SPF grade Sprague-Dawley (SD) female rats with a body weight of 160-180 g were selected for subcutaneous tumor preparation. Another 50 SD rats (half male and half female) with body weight of (209±19) g were used for experiment groups. All 56 SD rats were provided by the China National Food and Drug Administration [qualification number: SCXK (Beijing) 2014-0013] and were raised at the SPF Animal Experimental Center of the Cancer Hospital of the Chinese Academy of Medical Sciences. Rats had free access to food and water during feeding, and litter was changed 2-3 times a week. The humidity was 50%-70% and the temperature was 20-25 ℃. The whole process of the experiment was carried out in accordance with the *Instructive Notions with Respect to Caring for Laboratory Animals* promulgated by the Ministry of Science and Technology of China in 2006 and the related animal ethics regulations.

The fifty rats were randomly divided into a blank group (10 rats), a sham operation group (10 rats) and a model preparation group (30 rats) according to the ratio of half male and half female. The rats in the model preparation group were randomly divided into a model group (10 rats), a moxibustion group (10 rats) and an infrared group (10 rats) after they were successfully modeled for the experiment.

1.2 Main instruments and reagents

6002B infrared therapeutic apparatus (Xuzhou Tianfei Electronic Equipment Co., Ltd., China); MB-530 microplate reader (Jinan Anchang Medical Equipment Co., Ltd., China); LD5-10B low speed centrifuge (Beijing Jingli, China); rat board (homemade); rat moxibustion frame (homemade); moxa stick of 0.7 cm in diameter and 12 cm in length (Changsha Moxa Medical Technology Co., Ltd., China); ultra-clean workbench; white blood cell counting plate; cryotube; Eppendorf microcentrifuge tube; electronic balance; vernier caliper; beaker; absorbable suture; sterile gauze.

Pentobarbital Sodium (Biotobbed); phosphate buffer saline (PBS) solution (provided by Cancer Hospital, Chinese Academy of Medical Sciences); VEGF and EGF enzyme-linked immunosorbent assay (ELISA) kits (R&D Company, USA); saline (Hunan Kangyuan Pharmaceutical Co., Ltd., China); penicillin (800 000 U powder injection, Huabei Pharmaceutical Group Co., Ltd., China).

1.3 Modeling method

Ascites-type Walker-256 cancer cells were provided by the Cancer Hospital of Chinese Academy of Medical Sciences. Rat gastric cancer model was made by orthotopic transplantation[3-4]: the Walker-256 cancer cell suspension (2×10^7 cells/mL) was injected into the abdominal cavity of 3 rats (160-180 g), 0.3 mL/rat, to produce cancerous ascites after 7 d; the cancer cells in ascites were collected and adjusted to a concentration of 5×10^6 cells/mL, and 0.2 mL was injected subcutaneously into both the left forelimb and right hindlimb in three rats with a body weight of 160-180 g; a subcutaneous solid tumor with a size of 2 cm×2 cm×2 cm was formed at the injection site after 7 d. The solid-tumor-bearing rats were sacrificed and the tumor tissues were cut into 5 mm×5 mm×5 mm with a razor blade, transplanted into the stomach wall of rat gastric antrum in the model group, following 0.2 mL/rat subcutaneous injection of penicillin (800 000 U dissolved in 2 mL physiological saline). After 7 d, the exploratory laparotomy was performed and found that tumors of about 1 cm in diameter in the planting area formed with a smooth surface, clear boundary and no obvious organ invasion.

1.4 Intervention method

1.4.1 Blank group

Rats in the blank group were raised normally, grasped and fixed without other treatment.

1.4.2 Sham operation group

The exploratory laparotomy, stimulation of the stomach wall, close abdomen, and subcutaneous injection of penicillin were performed as in the model group, but the tumor tissue was not transplanted; the rats were grasped and fixed without other treatment during the intervention.

1.4.3 Model group

Rats only received modeling with grasping during the intervention treatment, and did not receive other treatment.

1.4.4 Moxibustion group

After successful modeling, suspended moxibustion was performed during the intervention treatment.

Acupoints: Two groups of acupoints were used. The first group included Zhongwan (CV 12), Guanyuan (CV 4) and bilateral Zusanli (ST 36); the second group included bilateral Pishu (BL 20) and Weishu (BL 21). Acupoints were located according to the *Experimental Acupuncture Science*[5], the commonly used animal acupoint positioning method combined with anthropomorphic method. The rat umbilicus is at the junction between the lower 1/4 and upper 3/4 of the line connecting the sternoclavicular and pubic symphysis; Zhongwan (CV 12) is located at the midpoint of the line connecting the umbilicus and the xiphoid process; Guanyuan (CV 4) is located at the intersection of the upper 3/5 and the lower 2/5 of the line connecting the umbilicus and the pubic symphysis, about 25 mm below the umbilicus; Zusanli (ST 36) is located at the posterolateral of the knee joint, about 5 mm below the capitulum fibulae; the Pishu (BL 20) is located between the ribs both sides under the spinous process of the 12th thoracic spine, and the Weishu (BL 21) is located between the ribs both sides under the spinous process of the 13th thoracic spine.

Methods: The suspended moxibustion using the pure moxa stick (7 mm in diameter and 12 cm in length) was applied alternately on the two sets of acupoints. The rat was placed on the rat board in a supine/prone position when receiving moxibustion. The moxa stick was fixed on the homemade moxibustion frame and ignited. The ignition point of the moxa stick was adjusted to 2-3 cm above the acupoints to make the skin temperature reach (42±2) ℃. The rat experimental thermometer was used for real-time temperature monitoring to adjust the distance between the ignition point and the acupoint in time.

Course of treatment: Intervention on the 2nd day after exploratory laparotomy, 20 min/time, once a day, for consecutive 21 d.

1.4.5 Infrared group

Location: The abdomen and back surface projection areas corresponding to the two groups of acupoints in the moxibustion group.

Methods: The projection areas corresponding to the acupoints were irradiated to ensure the skin temperature at (42±2) ℃ using the 6002B infrared therapeutic apparatus (short-wave infrared light of 0.4-3.0 μm, intensity of level 1, light spot diameter of 3 cm). The rat experimental thermometer was used to monitor the real-time temperature for adjusting the distance between the infrared probe and the irradiated areas.

Course of treatment: The intervention was conducted on the 2nd day after exploratory laparotomy, 20 min/time, once a day, continuously for 21 d.

1.5 Observation items and detection methods

1.5.1 Rat body weight measurement

All rats hade free access to food and water, and the rats were observed daily for flexibility, coat color, food intake and body weight. The body weight of rats in each group was measured every 4 d from the day of modeling.

1.5.2 Tumor volume and tumor growth inhibition rate after moxibustion

After the intervention treatment, the numbers of gastric tumor bearing rats in the model group, moxibustion group and infrared group were recorded, and the gastric tumor was completely removed. Tumor weight and tumor volume were obtained. Tumor volume (V) = $(\pi/6) \times [(a + b)/2]^3$ (a and b referred to the largest diameter and the smallest diameter of the tumor mass, respectively). Tumor inhibition rate (IR) = [1 − (Average tumor weight of the moxibustion group or the infrared group ÷ Average tumor weight of the model group)] × 100%.

1.5.3 Metastatic status of tumor cells

The tumor cell metastatic status was explored by opening the abdomen and recorded at the end of the intervention.

1.5.4 Detection of VEGF and EGF expressions in gastric tumor tissues

After the intervention treatment of rats in each group, the gastric tumor tissue was removed together with some gastric tissues (a piece of stomach tissue was removed instead in the blank group and the sham operation group) and stored in the liquid nitrogen after PBS rinse. During the test, a small piece of tumor tissue was homogenized at 4 ℃, centrifuged at 2 000 r/min for 10 min to collect the supernatant. The expressions of VEGF and EGF in rat gastric tumor tissues were detected by ELISA strictly according to the instructions.

1.6 Statistical method

Statistical processing was performed using SPSS 21.0 software. All measurement data fitting normal distribution were expressed as mean ± standard deviation ($\bar{x}\pm s$). Multiple group comparison was performed by one-way analysis of variance; the least significant difference (LSD) test was used to compare the data with variance homogeneity between groups. The Games-Howell test was used for variance heterogeneity data. The rank sum test (Kruskal-Wallis) was used if the normal distribution was not fit. The difference was statistically significant at $P<0.05$.

2 Results

2.1 Comparison of rat body weight

On the day of tumor tissue transplantation, there was no significant difference in rat body weight among the groups ($P>0.05$). On the 8th day of gastric tumor tissue transplantation, the body weight of each modeled rat was significantly lower than that in the blank group, and the difference was statistically significant ($P<0.05$); there was no significant difference between the moxibustion group and the model group ($P>0.05$). By the middle and late stages of intervention, compared with the model group, the body weight of the moxibustion group increased significantly (both $P<0.05$), (Table 1).

Table 1. Comparison of body weight changes in each group ($\bar{x}\pm s$, g)

Group	*n*	Day 1	Day 8	Day 20	Day 28
Blank group	10	222.5±19.43	250.4±39.00	269.7±58.59	279.4±67.79
Sham operation	10	230.1±15.09	256.2±45.60	263.7±58.39	275.5±61.65
Model	10	194.4±8.49	181.9±4.910 1)	169.1±4.01	171.9±6.98
Moxibustion	10	197.5±8.01	188.6±20.41 1)	211.5±20.53 2)	239.7±39.72 2)
Infrared	10	199.8±3.70	186.9±15.80 1)	180.6±14.57	172.6±8.29

Note: Day 1 was the day of tumor tissue transplantation; Day 8 was the second day after exploratory laparotomy; Day 20 was the mid-term of intervention; Day 28 was the end of intervention. Compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$

2.2 Comparison of tumor volume and tumor growth inhibition rate after intervention

After intervention, the number of tumor-bearing rats was 7 in the model group, 3 in the moxibustion group and 5 in the infrared group. Compared with the model group, the tumor volumes of the moxibustion group and the infrared group were significantly reduced ($P<0.05$). There was no significant difference in the tumor volume between the moxibustion group and the infrared group ($P>0.05$). The tumor growth inhibition rate was 41.89% in the moxibustion group and 28.09% in the infrared group. The difference between the two groups was statistically significant ($P<0.05$). See Table 2 for details.

Table 2. Comparison of tumor volume and tumor growth inhibition rate in each group ($\bar{x}\pm s$, point)

Group	*n*	Tumor volume ($\bar{x}\pm s$, mm^3)	Tumor growth inhibition rate (%)
Model	7	1353.93±265.76	/
Moxibustion	3	786.81±158.12 1)	41.89 2)
Infrared	5	973.61±288.41 1)	28.09

Note: Compared with the model group, 1) $P<0.05$; compared with the infrared group, 2) $P<0.05$

2.3 Comparison of tumor cell metastasis after intervention

After treatment, the exploratory laparotomy was carried out. The model group, the moxibustion group and the infrared group all appeared tumor cell metastasis, but the number of metastatic cases in the moxibustion group was less than that in the model and the infrared groups. See Table 3 for details.

Table 3. Comparison of rat tumor cell metastasis in each group (case)

Group	*n*	Metastatic site			
		Ascites	Liver	Intestine	Spleen
Model	10	1	1	1	1
Moxibustion	10	1	0	0	0
Infrared	10	3	0	0	3

2.4 Comparing the VEGF and EGF expressions in rat tumor tissues

Comparison of the VEGF level in tumor tissues: compared with the blank group, the VEGF level in the model group was increased ($P<0.05$); compared with the model group, the VEGF levels in the moxibustion group and the infrared group were significantly decreased (all $P<0.05$). There was no significant difference between the moxibustion group and the infrared group ($P>0.05$). Comparison of the EGF level in tumor tissues: compared with the blank group, the EGF level in the model group was decreased ($P<0.05$); compared with the model group, the EGF levels in the moxibustion group and the infrared group were significantly increased ($P<0.05$); there was no significant difference between the moxibustion group and the infrared group ($P>0.05$). See Table 4 for details.

Table 4. Comparing the VEGF and EGF levels in tumor tissues of each group ($\bar{x} \pm s$)

Group	*n*	VEGF (μg/L)	EGF (pg/mL)
Blank	10	1424.09±656.04	1104.48±408.44
Sham operation	10	1528.33±509.93	1066.23±385.46
Model	10	2881.75±891.89$^{1)}$	177.03±90.24$^{1)}$
Moxibustion	10	1307.00±576.11$^{2)}$	1123.91±486.63$^{2)}$
Infrared	10	1496.01±701.56$^{2)}$	759.30±380.01$^{2)}$

Note: Compared with the blank group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$

3 Discussion

Chinese medicine believes that the cause of tumor is 'the accumulation of qi leading to mass'. *Zhen Jiu Da Cheng* (*Complete Compendium of Acupuncture and Moxibustion*) points out that moxibustion can activate meridian qi and blood, thus playing a role in eliminating phlegm and stasis, stimulating qi to promote water, replenishing qi and blood in the Spleen and Stomach Meridians, and eliminating masses. As a new treatment method for cancer, moxibustion is still under exploration. Clinical studies have shown that moxibustion can improve the clinical symptoms of fatigue, poor appetite, abdominal distension, and weight loss in patients with gastric cancer, improve the quality of life and prolong the survival of patients with advanced gastric cancer[6-7]. Moxibustion at Shenque (CV 8) showed anti-cancer efficacy by inhibition of lymphatic metastasis[8]. Moxibustion is a treatment by burning moxa. The warming effect of burning is an important factor in its therapeutic effect[9]. Moxa produces a physical infrared ray that is very effective and suitable for the body when it is burned, and its radiant energy spectrum is between 0.8 μ and 5.6 μ. This indicates that moxibustion not only has thermotherapy, but also includes the pharmacological effects of near-infrared and moxa burning. Generally, far infrared rays act on the superficial parts of the human body to diffuse heat by conduction; while the wavelength of near-infrared rays is shorter than that of the far-infrared rays, and can directly penetrate into the deep tissues with strong energy. This experiment was carried out by moxibustion at Zusanli (ST 36), Zhongwan (CV 12), Guanyuan (CV 8), Pishu (BL 20) and Weishu (BL 21), which was compared with infrared radiation, to observe the effect of moxibustion on gastric tumor-bearing rats.

The initial tumor growth depends on the blood supply obtained from the original surrounding blood vessels. The blood supply of the original blood vessels is insufficient to meet the further need when the tumor grows, therefore, the new blood vessels allow the tumor to grow rapidly, meanwhile infiltrate and metastasize into the surrounding tissues. VEGF is one of the most typical tumor microangiogenesis stimulating factors. Studies have shown that[10-11], VEGF is highly expressed in most tumors and positively correlated with tumor proliferation, invasion and metastasis. Lack of oxygen inside the tumor is the reason for VEGF up-regulation. Therefore, the tumor hypoxia microenvironment can be improved by reducing the VEGF mRNA expression, the target gene of hypoxia inducible factor-1α (HIF-1α), thereby inhibiting tumor angiogenesis and exerting anti-tumor effect[12]. The cytokines of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) play important roles in the HIF-1α/VEGF pathway. Studies have shown that[13-14], moxibustion with seed-sized moxa cone can reduce the expressions of IL-1β, TNF-α and VEGF in tumor patients, regulate the body's inflammatory microenvironment, thereby improving the hypercoagulability of the tumor, inhibiting tumor growth and reducing death. Studies have shown that[15], thermotherapy can effectively reduce the VEGF expression, reduce tumor angiogenesis, inhibit tumor proliferation and metastasis. The results of this study showed that compared with the blank group, the VEGF level in the tumor tissue of the model group was increased ($P<0.05$). Compared with the model group, the VEGF level in the tumor tissues of the infrared group was decreased ($P<0.05$), which may be related to the infrared thermotherapy; the VEGF level in the tumor tissue of the moxibustion group was decreased ($P<0.05$), suggesting that the warming effect and pharmacological action of moxibustion can also effectively reduce the VEGF expression and inhibit the tumor growth, invasion and metastasis in gastric tumor-bearing rats.

EGF is a powerful keratinocyte division growth factor that mediates the dynamic balance of EGF receptor (EGFR) in maintaining proliferation and apoptosis of various tissue cells. As a tumor-promoting factor in the pathological condition, it promotes the progression of precancerous lesions and angiogenesis, while inhibiting apoptosis. EGF can also regulate cell defense and gastrointestinal secretion, promote intestinal epithelial development, and inhibit gastric acid and pepsin secretion[16]. EGF has a nutritional effect on the development of the digestive tract and promotes the repair of damaged gastric epithelial cells[17]. In this study, we found that the EGF level in tumor tissues was lower than that in the blank group (P<0.05), which may be related to the differentiation degree and characteristics difference of gastric cancer. After moxibustion, the EGF level in tumor tissues was increased (P<0.05). This study showed that VEGF was highly expressed in tumor tissues of gastric tumor-bearing rats, but it did not show a positive correlation between EGF and VEGF. Whether there is correlation between EGF expression and gastric cancer lesions, and whether EGF expression can be used as an independent indicator for predicting gastric cancer need to be further investigated.

In summary, moxibustion has a certain effect on increasing the body weight in gastric tumor-bearing rats, and the moxibution group is superior to the model group in tumor growth inhibition rate and tumor metastasis. Its inhibitory effect may be related to the regulation of the VEGF and EGF expressions in tumor tissues of gastric-bearing tumor rats. However, the specific mechanism and its correlation with tumors still need to be further explored. However, whether the higher number of cases of tumor metastasis in the infrared group is only related to the infrared irradiation or together with the depth of infrared irradiation, needs to be further explored. The results of this study suggest that moxibustion has an advantage in inhibiting tumor metastasis, and it is of great significance to study its mechanism of anti-tumor metastasis. The insufficiency of this experiment is that the inhibitory effect of moxibustion on tumors was only explored targeting the tumor stimulating factor, which is only one possibility in the prevention and treatment of tumors by moxibustion. Future studies should increase the sample size of experimental animals, increase the indicators of angiogenesis inhibitors, and further prove the anti-cancer effect of moxibustion on gastric tumor-bearing rats and its relationship with tumor metastasis.

Conflict of Interest

There was no potential conflict of interest in this article.

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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