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Original Research

Effects of herbal medicine on human uterine tumor-bearing nude mice

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ABSTRACT

Aim: Uterine leiomyomas are the most common benign uterine neoplasms associated with significant morbidity. Herbal formulas capable of restoring yin-yang balance by dispersing blood stasis may be useful for managing fibroid symptoms. **Materials and Methods:** In this study, the antitumor properties of three herbs *viz.*, *Trogopterus xanthipes* Milen-Edwards, *Paeonia lactiflora* Pallas, and *Ulmus davidiana* Planch were evaluated in nude mice injected intravenously with human malignant myomas. Tumor fragments were xenografted subcutaneously through a flank incision in female mice. The mice entered the study for 8 weeks when their tumors reached the threshold volume (260 mm^3). The mice were randomly allocated to receive subcutaneous injections of normal saline (Group 1; negative control), *P. lactiflora* Pallas (Group 2), *U. davidiana* Planch (Group 3), *T. xanthipes* Milen-Edwards (Group 4), and intravenous injections of paclitaxel (Group 5; positive control). The weight and tumor volume were measured, followed by histopathology. **Results:** A few cases of abdominal distention and death were observed in the negative control group. Furthermore, a considerable enlargement of the liver and spleen was observed in the negative control group at autopsy with a gradual increase in body weight during the experiment. The mean tumor volume which increased in negative control mice reduced in mice treated with herbal remedies or paclitaxel from day 14 onwards ($P < 0.05$). The degree of necrosis and apoptosis induction from herbal treatments was similar to that of paclitaxel. **Conclusion:** Collectively, three herbs *viz.*, *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch were able to induce necrosis and apoptosis of uterine leiomyoma cells, proving antitumor properties against uterine fibroids.

KEY WORDS: Leiomyomas, *Paeonia lactiflora* Pallas, *Trogopterus xanthipes* Milen-Edwards, tumor, *Ulmus davidiana* Planch

INTRODUCTION

Uterine fibroids, also known as leiomyomas, myomas, or fibromyomas, are the most common benign uterine neoplasms associated with significant morbidity of nearly 40% in women during their reproductive years [1]. Typical symptoms related to uterine fibroids, including abnormal menstrual bleeding, dyspareunia, non-cyclic pelvic pain and pressure, infertility, and repeated miscarriages, can compromise the quality of life and may affect fertility and pregnancy outcomes. In fact, an increase in pregnancy loss and reduction in pregnancy and live birth rates have been reported in recent analyses of symptomatic women with intramural fibroids [2]. More than half of symptomatic women surveyed internationally reported a negative impact on their quality of life due to a general impairment in sexual life, work performance, family relationship, and housekeeping [3].

Although the etiology and biology of uterine fibroids are poorly understood, a few risk factors have been identified for the development and growth of leiomyomas, such as ethnicity, nulliparity, genetics, and hormonal factors [4]. The classical neoplastic transformation of myometrium to leiomyoma likely involves somatic mutations of normal myometrium and the complex interactions of steroid hormones with local factors, such as growth and apoptosis-related factors [5]. There is increasing evidence to suggest that estradiol upregulates epidermal growth factor (EGF) receptor, transforming growth factor-β (TGF-β), and platelet-derived growth factor but downregulates p53 protein in leiomyoma cells [6]. However, progesterone may contribute to leiomyoma cell growth by augmenting EGF, TGF-β, and Bcl-2 protein, and inhibiting insulin-like growth factor and tumor necrosis factor [6]. While the effects of progesterone on fibroid growth have been suggested to be dual (i.e., stimulatory and inhibitory), strong evidence supports the role of estradiol in promoting leiomyoma tumorigenesis and growth.

The management of symptomatic fibroids has been performed traditionally with surgeries such as myomectomy or hysterectomy. Another option of treatment is uterine artery embolization during which the blood vessels to the uterus are blocked for fibroid shrinkage [7]. The pharmacological choice of gonadotropin-releasing hormone agonists may also shrink fibroids and control abnormal bleeding [4]. However, alternative

herbal remedies have been proposed to control uterine fibroid-related symptoms.

Herbal remedies for fibroids are common in complementary medicine. In a 6-month pilot study, herb, acupuncture, and mind-body healing treatments were found to shrink fibroids, increase patient satisfaction, and reduce bothersome symptoms [8]. According to the traditional Chinese medicine theory, uterine fibroids are caused by yin-yang imbalance in the body, which can be interpreted as disturbances in the endocrine system and blood circulation. Herbal formulas classified as being capable of restoring yin-yang balance by dispersing blood stasis may be useful in the management of fibroid symptoms. The Hyul-Boo-Chook-Ur-Tang (Xue-Fu-Zhu-Yu-Tang in Chinese), which contains a decoction of 13 specific herbs in Korean medicine, has been used for a long time to treat uterine fibroids and has appeared in Dongui Bogam, a classic Korean medical book of Oriental medicine that was first published in 1613 [9].

The effects of individual herbs in the Hyul-Boo-Chook-Ur-Tang are not well studied; therefore, this current study was performed to determine reliable treatment alternatives for uterine fibroids. Three major effective herbs out of the 13 ingredients in the Hyul-Boo-Chook-Ur-Tang, according to Dongui Bogam, were selected to determine their effects in nude mice-bearing human fibroid tumors. *Paeonia lactiflora* Pallas (radix) was traditionally used to tonify and purify the blood. *Ulmus davidiana* Planch (radix) was used for purifying blood and has long been known to have anti-inflammatory properties. *Trogopterus xanthipes* Milen-Edwards (feces) has been prescribed for alleviating pain, promoting blood circulation, and resolving blood stasis to arrest bleeding [10].

The aim of our study was to determine whether *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch have any antitumor properties against uterine fibroids. In the present controlled study, the nude mice were used as an *in vivo* tumor model for the transplantation and engraftment of leiomyomal cells to investigate the effect of herbs on clinical signs, body weight, tumor volume, and cell death.

MATERIALS AND METHODS

Five-week-old female BALB/C nude mice (n = 60, 14-19 g) were purchased from Central Lab. Animal, Inc. (Seoul, Korea). The mice were maintained under specific-pathogen-free conditions at the Experimental Animal Center at Biotoxtech Co., Ltd. (Ochang, Korea). The experiment was performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee at Biotoxtech Co., Ltd. (Ochang, Korea); the permit number was 101181.

The mice were held for 1 week after arrival to allow them to acclimate, and they had access to food and water *ad libitum*. The leiomyomal cells utilized in the current study were derived from human malignant myomas that were obtained at surgery from women (range, 25-55 years) undergoing myomectomy. The female nude mice were administered an intravenous injection of 1×10^7 leiomyomal cells and were euthanized 3 weeks later. Several well-isolated tumor colonies were harvested by excising the tumor-bearing tissues. Then, tumor colonies were aseptically dissected into 2 mm^3 sections.

For tumor xenograft, the mice were sedated by light isoflurane (Choongwae Pharma Corp., Seoul, Korea) anesthesia, and tumor colonies were implanted subcutaneously with an 11-gauge trocar inserted through a flank incision. The trocar was slowly withdrawn, and the incision was closed using an interrupted Vicryl suture for the skin. All manipulations were conducted under aseptic conditions using a laminar flow hood. All animals tolerated the procedure well.

After implantation, the mice were inspected daily for body weight loss, general clinical condition, and tumor formation. The tumor grew in the right flank area near the back of the mice. Animals were palpated for tumors twice weekly and entered into the study when the tumors reached an average volume of ~260 mm^3.

The mice were randomized into the following 5 groups (10 mice per group): Group 1, normal saline injected subcutaneously (negative control; 0.2 mL/day); Group 2, *P. lactiflora* Pallas injected subcutaneously (10 mg/kg/day); Group 3, *U. davidiana* Planch injected subcutaneously (10 mg/kg/day); Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously (10 mg/kg/day); Group 5, paclitaxel injected intravenously (positive control; 5 mg/kg; Bristol-Myers Squibb Co, Princeton, NJ, USA). The concentration of paclitaxel used in this study has minimal effects on mouse morbidity [11], which was further adjusted considering the dose of each herb. Mice in Groups 1-4 were injected once per day for 8 weeks, whereas those animals in Group 5 were injected twice per week for 8 weeks.

On a weekly basis, the body weight and tumor size of the mice were measured. The maximal length (L) and perpendicular width (W) of the tumor were measured using a digital caliper, and the tumor volume was calculated using the following formula: Tumor volume (mm^3) = L (mm) × W^2 (mm^2) × ½. Changes in tumor volume were used as an overall indicator of antitumor efficacy. At the end of the experiment, autopsies were performed in the mice, and tumors were excised and weighed. In addition, the collected tumors were placed in phosphate-buffered 4% formalin for 16 hours at room temperature and embedded in paraffin. The obtained tissue sections (4.5 μm) underwent staining with hematoxylin-eosin and TUNEL for evaluating necrosis and apoptosis grade, respectively, before microscopic examinations.

The data were presented as the means ± standard deviations. The difference in the tumor growth rate among the 5 groups of nude mice was determined by repeated-measures analysis of variance. Differences with a $P < 0.05$ were considered significant.

RESULTS

Some clinical abnormalities (i.e., abdominal distention and death) were observed in a few cases in the negative control group. In addition, a considerable enlargement of the liver and spleen was observed at autopsy. Several large grayish-white bulging nodules were visualized in the spleen. The liver appeared edematous with yellow discoloration. Coloring of the skin, skin induration, and abdominal distention were observed in mice injected with herbal remedies. Only abdominal distention was reported in the positive control group. The mice in the negative control group showed a gradual increase in body weight at least until 5 weeks of the experiment, which was probably due to an enlarged liver or spleen [Figure 1]. Afterward, the body weight of mice in both Groups 3 and 4 increased significantly compared to the negative control group. However, the positive control group and Group 2 (i.e., *P. lactiflora* Pallas-injected group) showed a completely opposite pattern. The metabolism inhibitory effects of *U. davidiana* Planch and *T. xanthipes* Milen-Edwards might have been associated with the declining effects of tumors during the later stage of the experiment. The treated animals showed significantly smaller tumors compared to the negative control group after 52 days ($P < 0.05$) [Figure 2]. The mean tumor volumes of Groups 1-5 animals were 2920 ± 1308 mm^3, 1765 ± 836 mm^3, 1807 ± 932 mm^3, 1717 ± 605 mm^3, and 1401 ± 745 mm^3, respectively. The mean tumor volume increased significantly from day 14 to day 52 in control mice but not treated mice. Furthermore, the tumor growth inhibition rates for Groups 1-5 were 0%, 20.6%, 21.7%, 20.3%, and 19.7%, respectively, confirming tumor regression in mice that were treated using herbs. When all tumors were further analyzed for necrosis and apoptosis using histopathological assay, the difference in tumor cell death was not significant among groups, but there was an uphill trend with treatment. The average percentage of apoptosis ([number of positive cells/number of total cells] × 100) for Groups 1-5 was 1.5%, 2.0%, 2.0%, 1.7%, and 1.7%, respectively [Figure 3]. The amount of apoptotic cell death varied with treatment; however, the percentage of apoptotic cells increased in tumors treated with herbs compared to the negative control tumors.

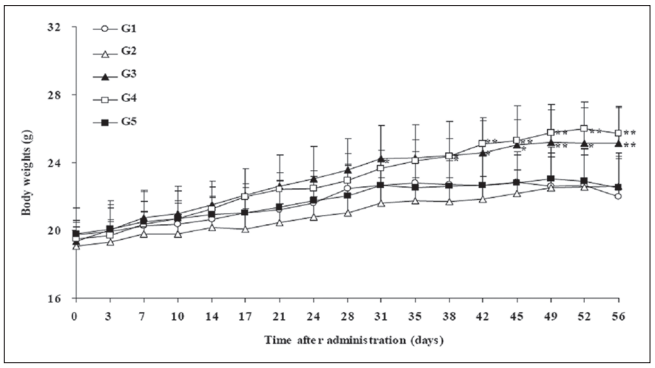

Figure 1: Effect of treatment on body weight (g) during the 8th week of experiment. Values at each point are presented as means ± standard deviations ($n = 10$). *$P < 0.05$ versus negative control, **$P < 0.01$ versus negative control. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

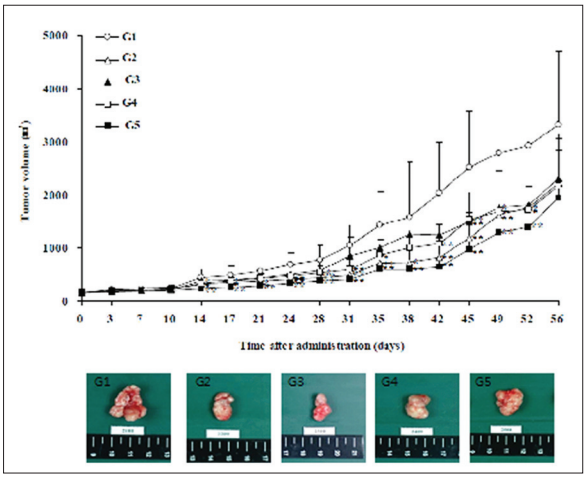

Figure 2: Effect of treatment on tumor volume (mm^3) during the 8th week of experiment. Each point in the curves represents the means ± standard deviations ($n = 10$). Representative tumor samples were obtained by implanting leiomyomal cells on the flank of the mice in each group. *$P < 0.05$ versus negative control, **$P < 0.01$ versus negative control. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

DISCUSSION

Herbal medicine is defined as the use of herbs to treat a wide range of disorders and enhance well-being. Herbs act on the body, similar to pharmaceutical drugs, and therefore, herbs should be treated with care. In Korea, most herbs are administered as a form of decoction. The effects of decoction can be manifested after total and final reaction by the constituent compounds when administered to humans. Herbal treatment for fibroids is a medical tradition in many countries worldwide [12].

Trogopterus feces (the feces of *T. xanthipes* Milen-Edwards) are used for the treatment of amenorrhea, dysmenorrhea, menstruation-related pain, and retained lochia due to stasis by promoting blood circulation and removing stasis. These effects may be due to fatty acid esters [13] and flavonoid glycosides [14] extracted from *Trogopterus* feces, which have been proven to possess anticoagulative activity. The anticancer activities of *Trogopterus* feces have been determined by deferred proliferation and more obvious changes of leiomyomal cells, which is probably through downregulation of TGF-β receptor 2 [15]. As a key profibrotic cytokine, elevated expressions of TGF-βs, their receptors, and related signaling pathways are common characteristics in leiomyoma [16]. Although we did not investigate the effect of each herb on TGF-β-related signaling pathways, some anticancerous effects of herbs observed in the present study might have been mediated

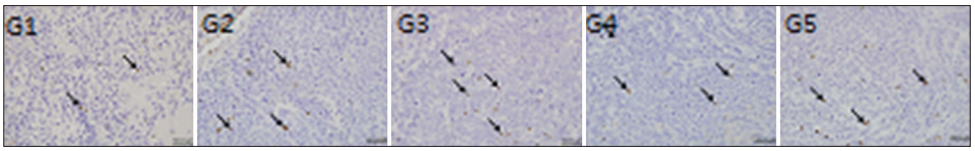

Figure 3: Representative micrograph showing terminal deoxytransferase-mediated dUTP nick-end labeling-stained apoptotic cells in each group. The arrows indicate terminal deoxytransferase-mediated dUTP nick-end labeling-positive apoptotic cells. G1: Group 1, normal saline injected subcutaneously (negative control); G2: Group 2, *P. lactiflora* Pallas injected subcutaneously; G3: Group 3, *Ulmus davidiana* Planch injected subcutaneously; G4: Group 4, *T. xanthipes* Milen-Edwards injected subcutaneously; G5: Group 5, paclitaxel injected intravenously (positive control)

through the downregulation of Smad and MAPK signaling pathways.

P. lactiflora Pallas combined with 4 other components has been reported as beneficial for reducing symptoms of uterine fibroids and shrinking uterine myomas [8,17]. Paeoniflorin, the main active constituent of *P. lactiflora* Pallas roots, has been proven to have potent antispasmodic, anticoagulative, analgesic, and anti-inflammatory activities [18]. Recently, another main monoterpene glycoside called albiflorin isolated from *P. lactiflora* Pallas was found to have similar anti-inflammatory effects to paeoniflorin [19].

U. davidiana Planch has been shown to have anti-inflammatory and anticancer activities based on its long history of clinical applications [20]. Its anti-inflammatory properties may be attributed to the inhibition of the cyclooxygenase pathway because prostaglandins are often highly produced in cancer and inflammation [21].

Histopathological analysis was performed to determine the induction of necrosis and apoptosis in each treatment and to further confirm the effects of herbal medicine at the cellular level. Interestingly, the level of the induction of necrosis and apoptosis from the herbal treatments was similar to that of paclitaxel. Paclitaxel has anticancer activity through a direct apoptotic effect, and it is commonly used to treat patients with gynecological cancer [22].

This study has several limitations. First, only three herbs of the Hyul-Boo-Chook-Ur-Tang have been examined for their potential antitumor properties against uterine fibroids. Therefore, the remaining herbs might have been overlooked despite considerable efforts to retrieve all relevant information of those herbs. Second, we did not explore any mechanisms to explain the potential antitumor properties of the herbs used. Another limitation of this study was the lack of a follow-up period, especially given that the tumor remained in the mice treated with herbs albeit the tumor growth was inhibited.

CONCLUSION

Three herbs *viz*., *T. xanthipes* Milen-Edwards, *P. lactiflora* Pallas, and *U. davidiana* Planch were used to induce necrosis and apoptosis of uterine leiomyoma cells proving antitumor properties against uterine fibroids. We predict that these herbs must have balanced the yin-yang ratio by dispersing blood stagnation from the body and alleviating inflammation.

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