Histopathological Evaluation of the Effectiveness of Oral Eppikajutsuto Treatment for Lymphatic Malformation

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Abbreviations: TJ-28, Eppikajutsuto; LM, lymphatic malformation; FIA, Freund's incomplete adjuvant; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; VEGF, vascular endothelial growth factor

Key Words: Lymphatic malformation; Model mouse; Japanese herbal medicine; Eppikajutsuto

ABSTRACT

Background: Lymphatic malformation (LM) is a congenital disease caused by lymphatic vessel malformation. Although standard therapies for LMs are sclerotherapy and/or surgical excision, a new therapy using Japanese herbal medicine Eppikajutsuto (TJ-28) has been recently reported as clinically effective. We aimed to experimentally confirm the therapeutic effectiveness of TJ-28 for LMs.

Methods: LM lesions were generated in the mesentery and peritoneum of mice by intraperitoneal injection of Freund's incomplete adjuvant. Mice with LMs were treated by gavage or dietary administration of TJ-28 for 2 months. Formalin-fixed paraffin-embedded tissue sections of mesentery and peritoneum tissues were histologically and immunohistochemically examined by focusing on lymph nodes and perinodal lymph vessels.

Results: Multiple Freund's incomplete adjuvant-associated foreign-body granulomas were formed in the mesentery and peritoneum, resulting in congestion of lymph fluid and dilatation of lymph vessels. The numbers and sizes of lymph nodes were not significantly different between TJ-28-treated and control groups. However, the luminal areas of lymphatic vessels were reduced significantly in the TJ-28 treatment group by both gavage and dietary administrations.

Conclusion: TJ-28 conspicuously reduced congestion of lymph fluid. This is the first histopathological evaluation of LM model mice to study the effectiveness of oral TJ-28 treatment.

Lymphatic malformations (LMs), which were previously called hygroma or lymphangioma, are caused by congenital malformation of lymphatic vessels that distribute invasively in various parts of the whole body. Standard therapeutic choices for LMs are sclerotherapy and/or surgical excision. Sclerotherapy with percutaneous injection of sclerosants (e.g., OK-432 and bleomycin) are effective for treatment of macro-cystic type LMs. It has been reported that antiangiogenetic therapy with sirolimus also proves to be effective in some children with LMs [1]. However, these treatments are ineffective for approximately 20% of LMs. Surgical resection potentially leads to a definitive cure of LMs, but it is often technically challenging in cases of LM lesions located close to vital structures [2].

Recently, several studies have reported the clinical effectiveness of oral administration of Eppikajutsuto (TJ-28), which is a Japanese herbal medicine manufactured by Tsumura & Co. (Tokyo, Japan), for treatment of LMs [2-5]. This treatment has attracted attention for LMs because of its minimal invasiveness, especially in pediatric patients. However, no experimental confirmation of the effectiveness has been reported, and the precise medicinal mechanisms remain to be clarified. Here, we report histopathological evaluation of TJ-28 treatment for LM using a lymphangioma model mouse established by Mancardi et al [6].

1. Materials and methods

This study was approved by the Institutional Ethical Review Board (Approval number: 1122) and conducted at the Laboratory Animal Research Center of Dokkyo Medical University.

1.1 LM Model Mice

Thirty-two C57BL/6 mice (6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). Each mouse was kept at room temperature in a 12-h light cycle and acclimated to the environment for 1 week. We induced LMs in the mesentery and peritoneum by intraperitoneal Freund's incomplete adjuvant (FIA) injection (0.2 mL emulsion) with a 2-week interval, according to the method of Mancardi et al [6].

1.2 Animal Experiment 1 (Gavage Administration of TJ-28)

Six mice with LMs were treated by gavage administration of 0.3 g/kg TJ-28 twice a day for 2 months (treatment group). Six mice also were subjected to gavage administration of water twice a day for 2 months (control group) (Figure 1a).

1.3 Animal Experiment 2 (Dietary Administration of TJ-28)

Twenty mice were divided into four groups, and 0 g/kg/day (control group), 0.3 g/kg/day (0.3 group), 1.5 g/kg/day (1.5 group), or 3.0 g/kg/day (3.0 group) of TJ-28 was administered orally to the five mice of each group for 2 months (Figure 1b).

1.4 Histopathological Evaluation of Lymph Vessels

At 2 months after starting oral administration of TJ-28, mice were euthanized by a pentobarbital overdose and dissected. Organs including mesentery and peritoneal tissues were fixed with 15% buffered formalin, embedded in paraffin, and processed into 4 μm-thick sections that were stained with hematoxylin and eosin or immunostained with an anti-mouse lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) antibody (DP3513P, Acris Antibodies, Germany), the Histofine SimpleStain system (Nichirei Bioscience, Tokyo, Japan), and diaminobenzidine (Nichirei Bioscience) after antigen retrieval using 0.01 mol/L citrate buffer (pH 6.0). The numbers of lymph nodes, lymph node areas, and lymph vessel areas were compared between the treatment and control groups. It is often hard to measure luminal dimensions of lymph vessels in mesenteric/peritoneal tissues unbiasedly because the mesentery and peritoneum are fat-rich tissues, and the interlobular septa, where lymph vessels were mainly localized, is distributed irregularly in the mesentery and peritoneum. To prevent sampling error and estimate the unbiased congestive condition of lymph fluid, summation of luminal areas of the lymphatic vessels within 1 mm from the capsule of lymph nodes (Figures 2a and 2b) was defined as lymph vessel areas because lymph fluid flows invariably into lymph nodes. Body weight changes before and after administration were also compared.

1.5 Statistical Analysis

The significance of differences was examined using Mann-Whitney's U-test. Results with a value of $P < 0.05$ were considered to be significant.

2. Results

Small white lesions within 2 mm were scattered in the mesentery and peritoneum, especially below the diaphragm, mesentery, and hepatic portal region of FIA-treated mice (Figure 3a). Representative histology of an experimentally generated LM lesion is shown in Figure 3b. The small white lesions identified by macroscopic examination consisted of foreign-body granulomas. Irregular fibrosis with dilated lymph vessels was found in the interlobular septa of mesenteric/peritoneal adipose tissues. No diffuse inflammation (pan-peritonitis) was noted in the peritoneal cavity and no ascites was found in both TJ-28-treated and control groups. There was also no remarkable pathological change in the liver, kidneys, heart, and lungs.

2.1 Animal Experiment 1 (Gavage Administration of TJ-28)

Data of body weight changes, numbers of lymph nodes, lymph node areas, and lymph vessel areas are summarized in Figure 4a. Body weight changes showed no significant difference (median +6.5 g, range +4.0 to +9.0 vs. +6.0 g, range +5.0 to +12.0, $P = 0.469$) between treatment and control groups. The numbers of isolated lymph nodes showed no significant difference (median 3.5, range 1 to 5 vs. 4.5, range 2 to 7, $P = 0.165$) between treatment and control groups. Lymph node areas also showed no statistically significant difference (median 0.89 mm^2, range 0.09 to 2.12 vs. 1.10 mm^2, range 0.75 to 2.32, $P = 0.221$) between treatment and control groups. However, the areas of lymphatic vessels were reduced significantly in the treatment group compared with the control group (median 1520.3 μm^2, range 683.3 to 2732.0 vs. 4877.9 μm^2, range 2373.1 to 14736.9, $P = 0.039$).

2.2 Animal Experiment 2 (Dietary Administration of TJ-28)

Data of body weight changes, numbers of lymph nodes, lymph node areas, and lymph vessel areas are summarized in Figure 4b and Supplementary Figures 1 and 2. Median of body weight changes of control, 0.3, 1.5, and 3.0 groups were +1.3 g (range +0.1 to +5.0), +2.5 g (range +2.5 to +7.5), +1.4 g (range +0.6 to +2.1), and +0.5 g (range -2.0 to +1.1), respectively. Body weight changes of control, 0.3, and 1.5 groups were increased during the experimental period, whereas those of 3.0 group were decreased. Body weight was significantly reduced in the 3.0 group compared with the 0.3 group ($P = 0.014$). Median numbers of lymph nodes in control, 0.3, 1.5, and 3.0 groups were 4 (range 1 to 4), 2 (1 to 3), 3 (2 to 6), and 2 (1 to 4), respectively. Numbers of lymph nodes were no significantly different among the four groups. Median areas of lymph nodes of control, 0.3, 1.5, and 3.0 groups were 0.35 mm^2 (range 0.31 to 0.70), 0.35 mm^2 (range 0.08 to 1.33), 0.20 mm^2 (range 0.12 to 0.33), and 0.28 mm^2 (range 0.11 to 0.37), respectively. Areas of lymph nodes were reduced significantly in the 1.5 group compared with the control group ($P = 0.027$). Median areas of lymphatic vessels in control, 0.3, 1.5, and 3.0 groups were 15781.1 μm^2 (range 7797.5 to 79906.0), 21558.6 μm^2 (range 10455.0 to 73320.4), 11243.8 μm^2 (range 5762.0 to 24646.7), and 7215.2 μm^2 (range 1619.6 to 8469.7). Areas of lymphatic vessels were reduced significantly in the 3.0 group compared with the control group ($P = 0.027$).

3. Discussion

Recently, several studies have suggested the clinical effectiveness of TJ-28 for treating LMs. Ogawa-Ochiai et al. reported the first case of a 2-year-old boy with mediastinal LM. The patient was treated with TJ-28 and Ogikenchuto [TJ-98, another Japanese herbal medicine supplied by Tsumura & Co. (Japan)] after OK-432 sclerotherapy. Displacement of the trachea was recovered and the neck mass had almost disappeared after 9 months [3]. Shinkai et al. reported a case of a 12-year-old girl with a large retroperitoneal LM, who received TJ-28 and surgery. They showed that surgery could be performed without complications because of reduction of the LM lesion by TJ-28 pretreatment [5]. Hashizume et al. reported eight pediatric patients with LMs, who received TJ-28 treatment. They described that mixed micro- and macro-cystic LMs were much more responsive to TJ-28 than macrocystic LMs because of less lymph fluid accumulation in mixed micro- and macro-cystic LMs than in macrocystic LMs [4]. However, these studies were clinical reports and did not provide experimental evidence for the therapeutic effectiveness of TJ-28. In this study, we experimentally confirmed the therapeutic effectiveness of TJ-28 for LMs using LM model mice reported by Mancardi et al. [6] and histopathology. White lesions were formed, particularly just below the diaphragm, mesentery, and hepatic portal region after intraperitoneal FIA injection. These findings were similar to those in the previous report [7]. Histological examination revealed dilatation of lymph vessels, suggesting congestion of lymph fluid in the mesentery and peritoneum. When enteral or dietary administration of TJ-28 was conducted in LM model mice, lymphatic vessels were reduced significantly compared with the control group.

Although we histopathologically confirmed the effectiveness of TJ-28 for LM treatment in this study, the precise mechanism was not clarified. TJ-28 contains six kinds of herbs: Sekko, Mao, Sojutsu, Taiso, Kanzo, and Shokyo. Sekko (*Gypsum fibrosum*) increases the expression of aquaporin 3 in skin and regulates skin moisture [8]. Mao (*Ephedrae herba*) is suggested to function as a suppressor of vascular endothelial growth factor (VEGF) activity by inhibiting prostaglandin E2 and cyclooxygenase protein synthesis [9]. VEGF is a key regulator of vascular permeability and lymphangiogenesis. Sojutsu (*Atractylodis Lanceae Rhizoma*) reduces the production of inflammation-related factors and suppresses the migration of macrophages [10]. Kanzo (*Glycyrrhiza glabra*) inhibits lipopolysaccharide-stimulated production of proinflammatory mediators such as nitric oxide, interleukin-1, and interleukin-6 [11]. These reports suggest that the herbal components in TJ-28 play an important role of inflammation and water regulation, resulting in reduction of lymph fluid from LM lesions. In animal experiment 2, we tried to verify the appropriate amount of TJ-28 to treat LM via dietary administration to LM model mice. Body weight changes was reduced significantly in the 3.0 group compared with the 0.3 group via dietary administration of TJ-28 for 2 months. Body weight tended to reduce as the concentration of TJ-28 increased (Figure S1), which was considered to be influenced by the anti-inflammatory and anti-edema effects of TJ-28. Food intake was almost the same among the four groups, but slightly tended to decrease as the TJ-28 concentration increased (Figure S2). It is possible that the herbal odor of TJ-28 affected its

palatability in mice. Further investigation is necessary to define which components in TJ-28 are pharmacologically and biologically effective for LM treatment.

4. Conclusion

This study experimentally provided histopathological evidence concerning the effectiveness of TJ-28 for LM treatment by a prospective controlled trial using LM model mice. Further vigorous investigation searching for the effective components in TJ-28 and its pharmacological kinetics might provide novel therapeutic strategies for LMs.

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Figure Legends

Figure 1. (a): Scheme of the experimental design of animal experiment 1. We generated LMs in the mesentery and peritoneum of mice by intraperitoneal injection of Freund's incomplete adjuvant (FIA). Six mice with LMs were treated by gavage administration of TJ-28 0.3 g/kg twice a day (treatment group). Six mice were subjected gavage administration of water twice a day (control group). (b)**:** Scheme of the experimental design of animal experiment 2. Twenty mice were divided into four groups of five mice each, and 0 g/kg, 0.3 g/kg, 1.5 g/kg, or 3.0 g/kg TJ-28 were administered orally for 2 months.

Figure 2. Microphotograph of a LYVE-1-immunostained peritoneal tissue section. Lymph vessels were colored brown. Nuclei were counterstained with hematoxylin. (a): Measurement of the lymphatic vessel area was conducted by summation of the luminal areas of lymphatic vessels located within 1 mm from the lymph nodes. (b): Dilated lymphatic vessels are noted (arrowheads).

Figure 3. Macroscopic (a) and microscopic (b) findings of mesenteric tissue from TJ-28-treated mice. White lesions (arrows), which consisted of foreign-body granulomas and caused lymphatic stagnation (arrowheads), were observed in the mesenteric area.

Figure 4. Comparison between TJ-28 treatment and control groups of animal experiment 1 (gavage administration) (a) and animal experiment 2 (dietary administration) (b). (a): Area of lymphatic vessels was significantly reduced by TJ-28 treatment. (b): Body weight change and area of lymphatic vessels were significantly reduced by TJ-28 treatment. 0.3, 0.3 group; 1,5, 1.5 group; 3.0, 3.0 group.