Effect of chiropractic manipulation on disrupted epithelium barrier and its mechanism of specialized pro-resolving mediators in a spleen-deficiency murine model

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OBJECTIVE: To investigate the influence of spleen deficiency on the epithelial barrier of jejunum and lungs in a rat model of spleen-deficiency and the effect and potential specialized pro-resolving mediators (SPMs) mechanism of chiropractic manipulation.

METHODS: Three-week-old male Sprague-Dawley rats were divided randomly into normal control group (n = 6), spleen-deficiency group (n = 5) and chiropractic group (n = 6). Spleen-deficiency model was induced in spleen-deficiency group and chiropractic group. Moreover, chiropractic manipulation was performed in chiropractic group. Four weeks later, systemic Th1/Th2 balance was evaluated by the ratio of plasma interferon (IFN)-γ/interleukin (IL)-4 levels by enzyme-linked immunosorbent assay (ELISA). Epithelial barrier integrity were assessed by the observation of morphological changes by hematoxylin-eosin staining and zonula occludens (ZO)-1 gene expressions by quantitative real time polymerase chain reaction in jejunum and lungs. Plasma resolvin D1 (RvD1) and lipoxin A4 (LXA4) levels were measures by ELISA for endogenous SPMs production. The levels of docosahexaenoic acid (DHA) and arachidonic acid (AA) in jejunum and lungs were also measured by HPLC-MS/MS.

RESULTS: Comparing with normal control group, spleen-deficiency group showed disrupted mucosa in jejunum, inflammatory condition in lungs, significantly decreased ratio of plasma IFN-γ/IL-4 levels and lower expressions of ZO-1 mRNA in both jejunum and lung tissues. Comparing with spleen-deficiency group, chiropractic group had less disrupted mucosa in jejunum and inflammatory condition in lungs, significantly increased systemic ratio of IFN-γ/IL-4 and expressions of ZO-1 mRNA in both jejunum and lung tissues. Chiropractic group had significantly enhanced plasma levels of RvD1 and LXA4, but had no significantly higher levels of DHA and AA in jejunum and lungs when comparing with spleen-deficiency group.

CONCLUSION: Spleen deficiency caused systemic Th1/Th2 imbalance towards Th2 polarization and epithelial barrier disruption in jejunum and lungs.
Chiropractic manipulation helped enhance endogenous SPMs production, which might be one of the action mechanisms of chiropractic manipulation on the improvement of epithelial barrier disruption.

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Keywords: Manipulation, chiropractic; Th1-Th2 balance; Epithelial barrier; Specialized pro-resolving mediators; Spleen deficiency

INTRODUCTION

Traditional Chinese Medicine (TCM) believes children are susceptible to spleen deficiency, which is considered as an important pathological basis of various diseases.1-3 A plethora of studies indicated that there were Th1/Th2 imbalance toward Th2 polarization and epithelial barrier disruption in jejunum in the murine model of spleen deficiency.4-6 Studies also reported that Th2 cytokines such as interleukin (IL) -4 played an important role in airway TJ disruption.7 TJs are located on the apical side of epithelial cells and mediate cell-cell adhesion, which have a critical role in the maintenance of epithelial barrier function against foreign invaders such as pathogens, particles, and allergens. Disrupted epithelial barriers may enhance the infiltration of foreign-substances into the subepithelia and resulting in the initiation or exacerbation of infection and allergic diseases.8-10 Our previous study showed spleen deficiency worsened the asthmatic inflammation and deterred the inflammation resolution in a murine model.11,12 Is it possible that spleen deficiency also negatively affects the epithelial barrier of lungs?

Chiropractic manipulation is a very popular and effective massage manipulation and traditional independent massage therapy in pediatric clinics. It has a good effect on invigorating the spleen and the prevention and treatment of pediatric diseases.13,14 However, its action mechanism is still unclear. Our previous study showed the application of chiropractic manipulation during spleen-deficiency period before asthmatic challenge could efficiently enhance LXA4 production and thus improve the inflammation resolution in asthmatic rats with spleen deficiency.15,16 LXA4 is one of endogenous specialized pro-resolving mediators (SPMs). SPMs are derived from dietary omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), which provide local control over the execution of an inflammatory response towards resolution, tissue protection and host defense. SPMs can also improve Th1/Th2 imbalance and epithelial barrier disruption.17-19 Insufficient endogenous SPMs production may deter the inflammation resolution and lead to chronic inflammation.20

To investigate whether spleen deficiency affect endogenous SPMs production and chiropractic manipulation help its production is useful to understand the essence of spleen deficiency and the mechanism of chiropractic manipulation. Therefore, this study focused on the influence of spleen deficiency on the epithelial barrier of jejunum and lungs and potential SPMs mechanism of chiropractic manipulation based on our previous studies.

MATERIALS AND METHODS

Animals

Three-week-old male specific pathogen-free Sprague-Dawley rats (purchased from Qinglongshan, Nanjing, China; Certificate of quality: No. 201721500) were housed in the Animal Laboratory in Nanjing University of Chinese Medicine at (24 ± 2) °C. All rats were fed with regular chow meeting the National Standard GB 14924.3-2001 (provided by Qinglongshan, Nanjing, China). Animal protocols were approved by Animal Care and Use Committee of Nanjing University of Chinese Medicine (No. 201805A004). The experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Spleen-deficiency model and group treatments

After acclimation for 3 d, all rats were divided randomly into normal control group (n = 6), spleen-deficiency group (n = 5) and chiropractic group (n = 6). Spleen-deficiency model was induced in spleen-deficiency group and chiropractic group modified based on described.21 Briefly, the rats were given Xiaochengqi decoction by gavage at 2 mL/100 g for 24 d once every other day. Meanwhile, the rats were not fed with any chow but drinking water freely on gavage day and were fed ad libitum on the next day. Chiropractic group were also performed with chiropractic manipulation once per day during modelling from Monday to Saturday for 28 d. Normal control group were given the same volume of double-distilled water by gavage with food and water ad libitum.

Xiaochengqi decoction, a kind of traditional Chinese formula, consisted of Houpo (Cortex Magnoliae officinalis), Zhishi (Fructus Citri aurantii immaturi), Dadhuang (Radix et Rhizoma Rhei) with the amount ratio of 3: 3: 2 for spleen-deficiency modelling. The herbs were put into cold water for 30 min, then decocted twice, mixed together according to regular protocol, and then condensed to 1 g/mL. The modelling was considered successfully if meeting the following criteria: (a) slow weight gain or weight loss; (b) less eating amount and more drinking volume; (c) loose stools; (d) lassitude or irritating; (e) fur with less luster. The procedure for chiropractic manipulation was as follows: The manipulator put on the disposal gloves. Then held the rat gently with the left palm and gently fixed a rat’s head with the thumb and index finger. Stroked the back skin slowly with the right index and...
middle fingers from the neck to the bottom for 3 times, and then pinching and pushing the skin from the bottom to the neck for 12 times, followed by pinching once and lifting three times for 5 repeats. The manipulating force was moderate to keep rats calm without screaming during the whole procedure. After 28 d, the rats were anesthetized with i.p. injections of 5 mL/kg 25% Urethane (Sigma, St. Louis, MO, USA, No. U2500). Blood was collected from abdominal aorta into tubes with ethylene diamine tetraacetic acid. Plasma was isolated by centrifugation at 3000 rpm for 20 min at 4 °C. The supernatant was collected and stored at −80 °C for later analysis. The middle lobe of the right lung was collected and put into a flask containing 4% buffered formalin solution for the pathological analysis, while the rest of the lung samples were collected, frozen immediately in liquid nitrogen and stored at −80 °C for later analysis.

Hematoxylin-eosin (HE) staining of the jejunum and lung tissues

The jejunum and lung tissues were collected and then immediately fixed in 4% buffered paraformaldehyde for 2 d at room temperature. The samples were then dehydrated and embedded in paraffin. Sections were then cut into 4-µm slices for routine HE staining (agents provided by Nanjing Jiancheng Bioengineering Institute, China). Images were obtained and analyzed under light microscopy (DP71/BX60, Olympus Corp., Tokyo, Japan).

Measurement of plasma interferon (IFN)-γ, IL-4, resolvin D1 (RvD1) and lipoxin A4 (LXA4) levels by enzyme-linked immunosorbent assay (ELISA)

The plasma levels of IFN-γ, IL-4, RvD1 and LXA4 were measured by ELISA according to the manufacturer’s protocol. ELISA kits (JEB-13301, JEB-13730, JEB-15403 and JEB-13867) were purchased from Nanjing Jin Yibai Biological Technology Co., Ltd. (Nanjing, China).

Measurement of zonula occludens (ZO)-1 mRNA expression by quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the jejunum and lung tissues with Trizol Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. RNA concentration and purity were determined by Eppendorf BioPhotometer. RNA quality was determined by electrophoresis. 2 µg of total RNA was used as template to synthesize cDNA with M-MLV Reverse Transcriptase kit (Thermo Fisher Scientific). RNA expression was measured by qRT-PCR with SYBR-Green method (Va-

zyme) according to the protocol. After normalization to the amount of endogenous GAPDH, the relative expression level of the target gene was calculated with the 2−∆∆CT method.

Primer was synthesized by Shanghai Sangon Biotechnology Company. The forward sequence for ZO-1 gene was 5′-GATGAGCGGGCTACCTTATTGA-3′, and the reverse sequence was 5′-TTTGTCGGGAGATCGTGACTG-3′. The forward sequence for GAPDH gene was 5′-ACAGCAACAGGGTGGGTGAGC-3′ and the reverse sequence was 5′-TTTGAGGTTGCAGCCAACCTT-3′.

Measurement of docosahexaenoic acid (DHA) and arachidonic acid (AA) levels

The levels of DHA and AA in jejunum and lung samples were measured with HPLC-MS/MS. 20 µL of the internal standards was added to each sample prior to extraction. Then, added 800 µL ice methanol into 10 mg of tissue samples and mixed with vortex for 5 min. The samples were centrifuged at 31876 × g for 10 min at 4 °C and collected the supernatant for MS analysis.

Conditions of chromatographic separation and MS detection: the levels of DHA and AA in jejunum and lung samples were analyzed with Thermo TSQ Vantage tandem mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) couple with an HPLC model U3000 apparatus (Dionex, San Jose, CA, USA). Chromatographic separation was achieved on a BDS Hypersil (2.1 mm × 100 mm, 2.4 µm) column at 40 °C. The mobile phase consisted of solvent A, water in 0.02 % formic acid, and solvent B, acetonitrile in 0.02 % formic acid. The mobile phases were eluted at 0.25 mL/min. The gradient was as follows: 35 % solvent B for 2.0 min, increased to 81 % at 6 min, held for the next 3 min, and then decreased to 10 % at 10 min, followed by a 3-min equilibration. The MS Vantage tandem mass spectrometer was operated with the following parameters: spray voltage, 3.5 kV; heated capillary, 300 °C; HESI probe, 350 °C; sheath gas flow, 45 psi; and auxiliary gas, 15 psi. The MRM transitions were adjusted by standards. DHA was 327.2 → 283.1 and AA was 303.2 → 259.2.

Statistical analyses

All data were expressed as mean ± standard deviation (x ± s). Statistical significance among groups was determined by one-way analysis of variance. All analyses and figures were performed with SPSS version 18.0 (IBM Inc., Armonk, NY, USA) and GraphPad Prism 6 (GraphPad Software Inc., La, Jolla, CA, USA) respectively. A P value < 0.05 was considered statistically significant.

RESULTS

Weight gain

Comparing with normal control group, spleen-deficien-
Chiropractic group gained significantly more weight than spleen-deficiency group \( (P < 0.05) \) (Table 1). In addition, spleen-deficiency group showed less eating amount, more drinking, loose stools with fetid smell, lassitude and fur with less luster, which met the criteria of spleen deficiency. Comparing with spleen-deficiency group, Chiropractic group showed improved condition of spleen deficiency.

**Morphological observation of the jejunum and lung tissues**

Morphological changes of jejunum and lungs were assessed by HE staining (Figure 1). Comparing with normal control group, spleen-deficiency group showed the disrupted mucosa in jejunum, which was characterized by shedding of mucosal epithelial cells, exposure of mucosal proper lamina and decreased length of villi and thickness of mucosa (Figure 1A, B). Moreover, comparing with normal control group, spleen-deficiency group also showed increased mucus in the airway, infiltration of inflammatory cells into perivascular, peribronchial and connective tissues in lungs (Figure 1D, E). Chiropractic group showed significantly improved condition in jejunum and lungs, including relative integrity of mucosal membrane, increasing length of villi and thickness of mucosa, less mucus in the airway and less infiltration of inflammatory cells in the lungs (Figure 1C, F).

**Ratio of plasma IFN-γ/IL-4 levels**

To assess the systemic Th1/Th2 balance, the levels of plasma IFN-γ/IL-4, the cytokines produced by Th1/Th2 cells respectively, were measured by ELISA and the ratio of plasma IFN-γ/IL-4 levels were calculated. Comparing with normal control group, spleen-deficiency group showed significantly decreased ratio of IFN-γ/IL-4 \( (P < 0.01) \). Chiropractic group showed significantly increased ratio of IFN-γ/IL-4 \( (P < 0.01) \) comparing with spleen-deficiency group (Figure 2).

**ZO-1 mRNA expressions in jejunum and lung tissues**

The normalized ZO-1 mRNA expressions were measured by qRT-PCR. Spleen-deficiency group showed significantly lower expressions of ZO-1 mRNA in both jejunum and lungs than normal control group \( (P < 0.01, P < 0.01) \), which indicated disrupted epithelial barriers in jejunum and lungs combined with morphological observation. ZO-1 mRNA expressions in both jejunum and lungs in chiropractic group were significantly higher than in spleen-deficiency group \( (P < 0.01, P < 0.01) \) (Figure 3).

**Plasma RvD1 and LXA4 levels**

Plasm levels of RvD1 and LXA4 in spleen-deficiency group did not show significantly higher comparing with normal control group \( (P > 0.05, P > 0.05) \). Comparing with spleen-deficiency group, chiropractic group showed significantly higher levels of RvD1 and LXA4 \( (P < 0.01, P < 0.05) \), which were also significantly higher than normal control group \( (P < 0.01, P < 0.01) \) (Figure 4).

### Table 1: Comparison of pre-modelling weight and pre-sacrifice weight-gain rate ( \( \bar{x} \pm s \) )

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pre-modelling weight (g)</th>
<th>Pre-sacrifice weight-gain rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>6</td>
<td>46±4</td>
<td>393±60</td>
</tr>
<tr>
<td>Spleen-deficiency</td>
<td>5</td>
<td>46±4</td>
<td>276±54*</td>
</tr>
<tr>
<td>Chiropractic</td>
<td>6</td>
<td>44±5</td>
<td>347±38*</td>
</tr>
</tbody>
</table>

Notes: normal control group: treated only with double-distilled water by gavage of 2 mL/100 g every other day; spleen deficiency group: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; chiropractic group: treated with Xiaochengqi decoction plus fasting and chiropractic manipulation. \( ^*P < 0.01 \) vs normal control group. \( ^*P < 0.05 \) vs spleen-deficiency group.

![Figure 1 Morphological changes in jejunum and lung of rats (hematoxylin-eosin staining, ×100)](image-url)

A-C: jejunum of normal control group, spleen-deficiency group and chiropractic group respectively; D-F: lung of normal control group, spleen-deficiency group and chiropractic group respectively. Normal control group: treated only with double-distilled water by gavage of 2 mL/100 g every other day; spleen deficiency group: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; chiropractic group: treated with Xiaochengqi decoction plus fasting and chiropractic manipulation.
NC: normal control group, treated only with double-distilled water by gavage of 2 mL/100 g every other day; SD: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; CP: chiropractic group, treated with Xiaochengqi decoction plus fasting and chiropractic manipulation. IFN-γ: interferon-γ, IL-4: interleukin IL-4. a P < 0.01 vs NC group; b P < 0.01 vs SD group.

Figure 2 Ratio of plasma IFN-γ/IL-4 level NC: normal control group, treated only with double-distilled water by gavage of 2 mL/100 g every other day; SD: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; CP: chiropractic group, treated with Xiaochengqi decoction plus fasting and chiropractic manipulation. IFN-γ: interferon-γ, IL-4: interleukin IL-4. a P < 0.01 vs NC group; b P < 0.01 vs SD group.

Figure 3 ZO-1 mRNA expressions in jejunum and lung tissues NC: Normal control group, treated only with double-distilled water by gavage of 2 mL/100 g every other day; SD: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; CP: chiropractic group, treated with Xiaochengqi decoction plus fasting and chiropractic manipulation. a P < 0.01 vs NC group; b P < 0.01 vs SD group. Presented data for quantitative real time polymerase chain reaction are representative of at least 3 separate and repeated experiments. ZO-1: zonula occludens-1.

Figure 4 Levels of plasma RvD1 and LXA4 NC: Normal control group, treated only with double-distilled water by gavage of 2 mL/100 g every other day; SD: treated with Xiaochengqi decoction by gavage of 2 mL/100 g and fasting every other day; CP: chiropractic group, treated with Xiaochengqi decoction plus fasting and chiropractic manipulation. a P < 0.01 vs CP group; b P < 0.01 vs NC group; c P < 0.05 vs CP group. SPMs: specialized pro-resolving mediators. RvD1: resolv D1; LXA4: lipoxin A4.

DHA and AA levels in jejunum and lung tissues

To detect the precursors of RvD1 and LXA4, levels of DHA and AA in jejunum and lung tissues were assessed by HPLC-MS/MS. Comparing with normal control group, spleen-deficiency group showed significantly lower level of AA in jejunum, DHA and AA in lung tissues (P < 0.01, P < 0.05, P < 0.05), despite not statistically lower level of DHA in jejunum (P > 0.05). Chiropractic group showed DHA and AA levels in jejunum and lung tissues were not significantly higher than spleen-deficiency group (P > 0.05, P > 0.05), but AA level in jejunum was not significantly lower than normal control group (P > 0.05) (Figure 5).

DISCUSSION

TJs have a critical role in the maintenance of epithelial barrier function, which are supported by adaptor proteins in the cytoplasm such as ZO proteins, which regulate junction assembly and selective paracellular permeability by signal transduction. The present study indicated systemic Th1/Th2 imbalance towards Th2 polarization and the disrupted epithelial barrier in jejunum in the spleen-deficiency rat model, which is consistent with previous reports. More importantly, this study also indicated that spleen deficiency induced inflammatory status and potential disrupted epithelial barrier in the lungs. To the best of our knowledge, the pathological state and epithelial barrier in the lungs in the spleen-deficiency model was the first time to investigate. IL-4, as a major Th2 cytokine, played an important role in TJs disruption, which help explain the disrupted epithelial barrier and consequently inflammatory status in lungs in this study. This study showed that spleen deficiency caused TJs disruption in the jejunum and lungs, which may increase the susceptibility and severity of infection and allergic reaction of digestive and respiratory systems. TCM theory believes that the spleen is the mother-organ of the lungs, which indicates the intimate relationship between them. In clinical investigation, spleen deficiency is the key pathological basis for common respiratory diseases among children such as recurrent respiratory tract infection and asthma.

Resolution of inflammation is initiated by an active class switch from pro-inflammatory lipid mediators, such as cysteinyl leukotrienes (CysLTs) and prostaglandins E (PGE_2), to the production of SPMs (LXA4 and PGE_2), which help explain the disruption of epithelial barrier function in jejunum and lung tissues. The increase of TJs permeability may be related to the enhanced production of CysLTs and PGE_2, which is also in line with previous reports. The increased levels of CysLTs and PGE_2 may also indicate that spleen deficiency induced inflammation in the spleen-deficiency rat model, which is consistent with previous reports. More importantly, this study also indicated that spleen deficiency induced inflammatory status and potential disrupted epithelial barrier in the lungs. To the best of our knowledge, the pathological state and epithelial barrier in the lungs in the spleen-deficiency model was the first time to investigate. IL-4, as a major Th2 cytokine, played an important role in TJs disruption, which help explain the disrupted epithelial barrier and consequently inflammatory status in lungs in this study. This study showed that spleen deficiency caused TJs disruption in the jejunum and lungs, which may increase the susceptibility and severity of infection and allergic reaction of digestive and respiratory systems. TCM theory believes that the spleen is the mother-organ of the lungs, which indicates the intimate relationship between them. In clinical investigation, spleen deficiency is the key pathological basis for common respiratory diseases among children such as recurrent respiratory tract infection and asthma.
sorbed majorly via duodenum and jejunum from diet. This study indicated that spleen deficiency also affected the levels of DHA and AA in jejunum and lungs, which may also play a role in the insufficient production of RvD1 and LXA4. Based on TCM theory, chiropractic manipulation is performed to stimulate back skin, Du vessel and Urinary Bladder meridian majorly involved. A plethora of clinical and experimental studies showed that chiropractic manipulation helped invigorate the spleen efficiently and had good effects on preventing-treating common pediatric diseases. The present study showed chiropractic manipulation could improve the systemic Th1/Th2 imbalance and epithelial barrier disruption in both jejunum and lungs. Meanwhile, chiropractic manipulation significantly enhanced systemic levels of RvD1 and LXA4. However, chiropractic manipulation didn’t significantly affect the levels of DHA and AA. Our previous study showed chiropractic manipulation could increase 15-LO and LXA4 levels in asthmatic rat with spleen deficiency, which could help deter the increased levels of CysLTs and PGE2. Combined with our previous study, this study results suggested that the enhanced production of RvD1 and LXA4 by chiropractic manipulation is related to the improved metabolism efficiency from DHA and AA into RvD1 and LXA4. However, to our surprise, the levels of DHA and AA were not enhanced with the improved epithelial barrier in jejunum by chiropractic manipulation. We speculated the enhanced metabolism from PUFA into SPMs might be a possible reason. In the future study, we need further investigate the detailed mechanism of chiropractic manipulation on the absorption of PUFAs and acting pathway of SPMs. In conclusion, the present study suggested that spleen deficiency caused systemic Th1/Th2 imbalance towards Th2 polarization and epithelial barrier disruption in jejunum and lungs. Chiropractic manipulation helped enhance the endogenous production of SPMs, which might be related to the improvement of the pathological conditions above in spleen deficiency.

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