



Original Article

The Effects of Pulsatilla Koreana NAKAI Pharmacopuncture Therapy on the Chunchu (ST25) for Dextran Sulfate Sodium-Induced Colitis in Mice



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ABSTRACT

Background: This research was performed to investigate the effects of Pulsatilla Koreana NAKAI pharmacopuncture (PPA) therapy on intestinal disease in rats with dextran sulfate sodium (DSS)-induced colitis.

Methods: The subjects were divided into five groups : A control group, saline group, pharmacopuncture group PPA1 (0.2 mg/ 1 kg/ 40 μ l), pharmacopuncture group PPA2 (0.5 mg/ 1 kg/ 40 μ l), and pharmacopuncture group PPA 3(1 mg/ 1 kg/ 40 μ l). The experimental model of colitis was induced by infection of dextran sulfate sodium (DSS) for eighteen days. After colitis was induced, PPA therapy was practiced on the Chunchu (ST25) once every two days for a total six times. Thereafter Disease Activity Index (DAI), colon length, damage to the colonic mucosa, body weight, IL-6, IL-10, IL-1 β , IFN- γ , TNF- α , TGF- β 1, IL-23 and IL-17 were measured.

Results: The results were as follows.

1. DAI was significantly decreased in the PPA groups.
2. Colon length was significantly increased in the PPA groups.
3. Damage of colonic mucosa was observed less in the PPA groups.
4. Body weight was significantly increased in the saline group and the PPA groups.
5. The PPA2 group showed a significant decrease in the intensity of IL-6, IL-1 β , IFN- γ and TNF- α levels and the mean of IL-23.
6. The PPA3 group showed a significant increase in the intensity of IL-10 and TGF- β 1 levels.
7. No significant differences were shown in the mean of IL-17.

Conclusion: These results suggest that PPA therapy on Chunchu (ST25) can be used as an effective treatment for inflammatory bowel disease.

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Introduction

Colitis, which causes chronic hemorrhagic diarrhea, abdominal pain, fever and weight loss due to chronic inflammation, is a rapidly growing disease with a recent increase in the incidence due to westernized food culture and lifestyle habits [1]. Although progression of inflammatory processes that improve and deteriorate affects the intestinal mucosa and increases the risk of developing colon cancer [2] the cause is not clear enough to estimate the psychological problems and immunological

mechanism such as infection and autoimmunity [3].

Although uncertain causes cause long-term use of anti-inflammatory drugs and surgical procedures, side effects such as osteoporosis, adrenal insufficiency, and excessive fluids due to steroid drugs are also serious [4]. Therefore, various experimental studies on alternatives are under way, and accordingly, studies and reports of Korean medicine are also active.

Studies on Korean medicine have been proceeded and reported by the method of Korean medicine, and studies using medicinal prescriptions such as Soyumjungjang-tang [5], Jiyutang [6],

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Hwangryunhaedoktang [7] and studies using prescription of single herb such as Auklandia Lappa [8], Mume Fructus [9], Orostachys japonicas [10]. On the other hand, there are only a handful of studies that have applied acupuncture and pharmacopuncture such as Five Transport Points of the Spleen Meridian [11], Sophorae Radix Pharmacopuncture [12].

The purpose of this study is to search for the treatment of colitis in Korean medicine based on the report on the therapeutic effects of the existing pharmacopuncture therapy application studies and taken advantage of stimulating directly the proximal abdominal acupoints and the pharmacopuncture therapy which can double the treatment efficiency by the double effect of acupuncture treatment and herbal medicine therapy [13]. We report the results of this study, because changes in Disease Activity Index (DAI), the length of colon, mucosal tissue of the colon, body weight, cytokine concentration and gene expression level by reverse transcription polymerase chain reaction (RT-PCR) were measured after administering the Pulsatilla pharmacopuncture therapy to the Chunchu (ST25) of rats with Dextran sulfate sodium (DSS)-induced colitis.

Materials and Methods

Animal

The mice of BALB/c origin (Samtako Bio Korea, Osan-si Korea) weighing about 20-23 g were adapted to the laboratory environment for 3 days or more while feeding solid feed (Samtako Bio Korea, Osan, Korea) and water in a habitat of constant temperature, humidity environment (room temperature $24 \pm 1^\circ\text{C}$, humidity $60 \pm 5\%$) and used in the experiment. Also, water and solid feeding were freely consumed Respectively.

Pulsatilla pharmacopuncture

After 200 g of Radix Pulsatilla (Radix Pulsatilla koreana, Nakai) produced in China was boiled. Water was evaporated with a rotary evaporator (Buchi, Flawi, Netherlands) and concentrated under reduced pressure. The concentrated sample was lyophilized at -80°C using a freeze dryer (Ilshin, Dongducheon, Korea) to obtain 15 g of Radix Pulsatilla koreana Nakai. This was diluted with physiological saline and adjusted to pH 7 with a pH meter (ORION, Bronshoj, USA). They were refrigerated and used for the procedure.

Induce colitis

Colitis was induced by ingesting drinking water containing 5% (w/v) dextran sulfate sodium (DSS, Sigma, Saintlouis, USA) for 18 days. Body weight, stool concentration, and presence or absence of stool were observed during the treatment for 6 times.

Experimental setup

The following groups were set up for the experiment. The normal group (normal, $n=5$) did not cause colitis and did not take Pulsatilla pharmacopuncture therapy. Control group (Control, $n=5$) did not undergo Pulsatilla pharmacopuncture therapy after inducing colitis with DSS. Saline group ($n=5$) was treated with physiological saline solution to the Chunchu (ST25) in place of Pulsatilla pharmacopuncture therapy after inducing colitis with DSS. Pulsatilla pharmacopuncture therapy 1, 2, and 3 groups (PPA1, PPA2, PPA3, $n=5$) were induced colitis with DSS and then were treated by Pulsatilla pharmacopuncture at a concentration

of 0.2 mg/ 1 kg/ 40 μl , 0.5 mg/ 1 kg/ 40 μl and 1 mg/ 1 kg/ 40 μl , respectively on Chunchu (ST25).

Locating acupoints and Pharmacopuncture therapy

The pharmacopuncture therapy was performed on both sides of the Chunchu (ST25). Chunchu (ST25) was determined in the following way. On the mice body, we divided the distance between the upper side of pubic symphysis and the lower side of xiphoid process by 13. The point 5/13 above the upper side pubic symphysis was defined as point A. A point and a midaxillary line were divided into 8 equal parts and horizontally 1/4 point at point A is Chunchu (ST25), corresponding to the human body. The treatment was 40 μl for total 6 times, once every 2 days from the 7th day of DSS administration. The solution was injected with insulin syringe (31 G \times 8 mm, BD, MD USA).

DAI

DAI is a clinical parameter that is a comprehensive functional measure similar to the subjective clinical symptoms observed when ulcerative colitis occurs in the human body and is calculated using the following formula (Table 1).

Table 1. Disease Activity Index.

Score	Clinical findings
1	No bleeding with normal stool
2	No bleeding with semiformal stool(+)
3	No bleeding with semiformal stool(++)
4	No bleeding with unformed stool
5	Fecal blood with pasty and unformed stool

Weight measurement

Weights of the mice were measured eight times (before inducing colitis, after inducing colitis and after treatment) during the experiment period using the electronic balance (CAS, Yangju, Korea).

Colonic length and histological measurement

After completion of the experiment, mice were sacrificed under respiratory anesthesia. After abdominal incision, they were harvested from the end of the large intestine to the cecum, and the length and degree of damage from the cecum to the end of colon were measured. 1 cm of tissue under the appendix and 1 cm of tissue above the distal end of the colon were fixed in bouin' solution. The fixed tissue was made paraffin blocks and cut into 6 μm intervals with a microtome, placed on a slide, stained according to H & E staining method, and observed with an optical microscope (Nikon 80i, Tokyo, Japan).

Total RNA isolation for RT-PCR

The harvested colon was rapidly frozen in liquid nitrogen and stored at -70°C until analysis. Total RNA was isolated by homogenization in precllys 24 (Bertin technologies, Montigny le Bretonneux France) with 800 μl Trizol Reagent (Lifetechnologies, Carlsbad, USA) in colon tissue (50 mg) and to the homogenate

was added 200 μ l of chloroform (Sigma, Saintlouis, USA), shaken for 15 seconds, mixed well, left at room temperature for 5 minutes, centrifuged at 4°C and 14,000 rpm for 5 minutes with centrifuges (Centrifuge 5415 R: Eppendorf, Wesseling Berzdorf, Germany) to remove the cell debris. 500 μ l of isopropanol (Sigma, Saintlouis USA) was added to the supernatant obtained by centrifugation, and the mixture was allowed to stand at room temperature for 5 minutes and was centrifuged at 14,000 rpm for 8 minutes at 4°C to get RNA pellet. Add refrigerated 70% ethanol and diethyl pyrocarbonate (DEPC) to the pellet and centrifuge at 7,500 rpm for 5 minutes at 4°C. Remove all but the pellet. After removing all but the pellet, the remaining ethanol was left to stand at room temperature for 10 minutes, dried, and then dissolved in DEPC-treated water. The purity and concentration of RNA were determined by reading the OD260 value from a spectrophotometer (Biophotometer: Eppendorf, Wesseling Berzdorf, Germany).

RT-PCR

5 μ g of isolated total RNA and 2.5 μ l of Oligo (dT), DEPC-treated water were added to RT premix (Bioneer, Daejeon, Korea) and 50 μ l cDNA was synthesized using Mastercycler gradient (Eppendorf, Wesseling- Berzdorf, Germany). cDNA was used as a template for PCR amplification. The housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control. The reverse transcription temperature cycle consisted of cDNA synthesis at 42°C for 1 hour, denaturation at 94°C for 5 minutes, and cooling at 4°C for 5 minutes. The PCR was amplified using a Mastercycler gradient (Eppendorf, Wesseling-Berzdorf, Germany) after adding 10pg of sense primer, 10pg of antisense primer and DEPC-treated water to PCR premix (iNtron, Seongnam-si Korea).

The DNA of the amplified IL-6, IL-10, IL-1 β , IFN- γ , TNF- α and TGF- β 1 was electrophoresed on 0.5% TBE buffer (80 mM Tris-HCL, 80 mM boric acid, 2 mM EDTA, pH 8.3) on 1.5% agarose gel containing Greenview nucleic acid gel stain (IO Rodeo, 1:10,000) at 100 V and observed. The images were taken using Image Station

(Samsung, Suwon, Korea) and measured with Alphaease FC StandAlone Software (Alpha Innotech, Sanleandro, USA).

Cytokine measurement by ELISA

After completion of the experiment, 500 μ l of 1X PBS was added to the harvested colon tissue (50 mg), homogenized in precellys 24 (Bertin technologies, Montigny le Bretonneux, France) and centrifuged (Centrifuge 5415 R: Eppendorf, Wesseling-Berzdorf, Germany) at 7,300 rpm for 5 minutes. The supernatant was separated and the supernatant was stored frozen until measurement.

Interleukin-17 (IL-17) measurement

IL-17 was measured using Mouse IL-17 Elisa Kit (Biomatik, willmington USA). Added 100 μ l of Mouse IL-17 Standard tissue sample to a microplate coated with IL-17, and the mixture tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 2 hours. After incubation, the plate was discarded without washing, 100 μ l of 1X Biotin-antibody was added, and the mixture was tapped with a plate cover (check cloudy state), mixed for 1 minute, and incubated at 37°C for 60 minutes. After washing three times with 400 μ l of wash buffer, 100 μ l of 1 \times HRP-avidin was added, and the mixture was tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 60 minutes. After washing 5 times with 400 μ l of wash buffer, 90 μ l of Tetramethylbenzidine(TMB) substrate was added, and the mixture was tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 30 minutes (dark state). After stopping the color reaction by adding 50 μ l of stop solution to the plate, Optical density (OD) was measured at 450 nm using a microplate spectrophotometer (Benchmark plus, Biorad, Hercules, USA).

IL-23 measurement

IL-23 was measured using Mouse IL-23 Elisa Kit (Biomatik, Willmington, USA). Added 100 μ l of Mouse IL-23 Standard tissue sample to a microplate coated with IL-23, and the mixture tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 2 hours. After incubation, the plate was discarded without washing, 100 μ l of 1X Biotin-antibody was added, and the mixture was tapped with a plate cover (Check cloudy state), mixed for 1 minute, and incubated at 37°C for 60 minutes. After washing three times with 400 μ l of wash buffer, 100 μ l of 1 \times HRP-avidin was added, and the mixture was tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 60 minutes. After washing 5 times with 400 μ l of wash buffer, 90 μ l of TMB substrate was added, and the mixture was tapped with a plate cover, mixed for 1 minute and incubated at 37°C for 30 minutes (dark state). After stopping the color reaction by adding 50 μ l of stop solution to the plate, Optical density (OD) was measured at 450 nm using a microplate spectrophotometer (Benchmark plus, Biorad, Hercules, USA).

Statistical processing

Experimental results were expressed as mean \pm SE. Among non-parametric methods, Mann-Whitney U test was used to observe statistical significance among the experimental groups with Window SPSS (version 21, SPSS).

Table 2. Primers Used for Polymerase Chain Reaction (PCR) Amplification.

Primer		Sequence
GAPDH	Forward	5'-ACTCCATCACCATCTTCCAG-3'
	Reverse	5'-CCTGCTTTCACCACCTCCTTG-3'
IL-6	Forward	5'-TGCTGGTGACAACAACGCC-3'
	Reverse	5'-GTACTCCAGAAGACCAGAGG-3'
IL-10	Forward	5'-TGCCTTCAGTCAAGTGAAGAC-3'
	Reverse	5'-AAACTCATTCATGGCCTTGTA-3'
IL-1 β	Forward	5'-ATGGCAACTGTTCTGAACTCAACT-3'
	Reverse	5'-CAGGACAGGTATAGATTCTTCTTT-3'
INF- γ	Forward	5'-AACGCTACACACTGCAATCTTGG-3'
	Reverse	5'-CTCGGATGAGCTCATTGAATGC-3'
TNF- α	Forward	5'-ATGAGCACAGAAAGCATGATC-3'
	Reverse	5'-TACAGGCTTGTCACCTGAAT-3'
TGF- β 1	Forward	5'-GCTTCAGACAGAACTCACT-3'
	Reverse	5'-GAACACTACTACATGCCATTAT-3'

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin ; INF, interferon; TGF, tumor necrosis factor; TNE, transforming growth factor.

Result

Effect on DAI

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on the DAI, The control group was 3.6 ± 0.33 (score) at the first treatment, 4.1 ± 0.24 (score) at the second, 4.3 ± 0.91 (score) at the third, 3.8 ± 0.44 (score) at the fourth, 3.1 ± 0.56 (score) and 3.7 ± 0.37 (score) at 6th treatment, respectively. All control group was significantly increased compared to normal group (*p*<0.05) (Table 3; Fig. 1).

Table 3. Changes of Disease Activity Index.

Group	Pharmacopuncture					
	1	2	3	4	5	6
Normal	1.0±0.00*	1.0±0.00*	1.0±0.00*	1.0±0.00*	1.0±0.00*	1.0±0.00*
Control	3.6±0.33	4.1±0.24	4.3±0.91	3.8±0.44	3.1±0.56	3.7±0.37
Saline	3.8±0.58	3.8±0.58	3.9±0.48	2.8±0.25	2.2±0.12	3.4±0.10
PPA1	3.0±0.27	3.0±0.27†	2.9±0.48	1.7±0.12†	2.0±0.16	1.9±0.10†
PPA2	3.3±0.30	3.5±0.32	3.7±0.25	2.3±0.41†	2.3±0.12	1.9±0.10†
PPA3	2.7±0.25	3.1±0.53	4.2±0.20	2.3±0.30†	2.4±0.46	1.8±0.12†

Normal, Group no treatment without 5% dextran sulfate sodium (DSS)-induced colitis.
 Control, Group no treatment with 5% DSS-induced colitis.
 Saline, Group treated with saline injection at ST25 after 5% DSS-induced colitis.
 PPA1, Group treated with Pulsatilla pharmacopuncture(0.2 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 PPA2, Group treated with Pulsatilla pharmacopuncture(0.5 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 PPA3, Group treated with Pulsatilla pharmacopuncture(1 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 Values are presented as mean ± SE.
 **p*<0.001.
 †*p*<0.05 compared with control.
 PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST25, Chunchu.

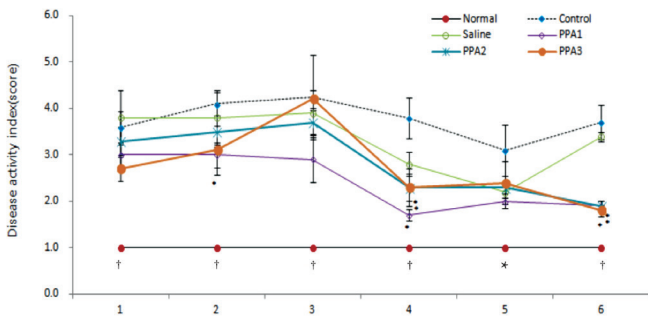


Fig. 1. Effect of Pharmacopuncture (Pulsatilla koreana, Nakai) at ST25 on clinical signs in DSS-induced colitis.

Normal, group no treatment without 5% dextran sulfate sodium (DSS)-induced colitis.
 Control, group no treatment with 5% DSS-induced colitis.
 Saline, group treated with saline injection at ST25 after 5% DSS-induced colitis.
 PPA1, group treated with pharmacopuncture (0.2 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 PPA2, group treated with pharmacopuncture (0.5 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 PPA3, group treated with pharmacopuncture (1 mg/ 1 kg/ 40 μl) at ST25 after 5% DSS-induced colitis.
 Values are presented as mean ± SE
 **p*<0.001
 †*p*<0.05 compared with control.
 PPA, Pulsatilla Koreana NAKAI pharmacopuncture.

Effect on the change of colon length

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on colon length, 9.4 ± 0.27 cm in the normal group, 6.3 ± 0.25 cm in the control group, 7.7 ± 0.57 cm in the saline group, 7.8 ± 0.31 cm in the PPA1 group, 7.3 ± 0.14 cm in the PPA2 group and 7.3 ± 0.11 cm in the PPA3 group respectively. PPA1, PPA2, and PPA3 groups showed a significant increase (*p*<0.05) compared to the control group (Figs 2, 3).

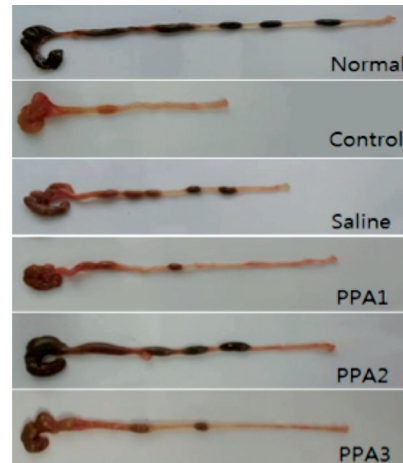


Fig. 2. The Representative samples of colons were shown. The colons were removed at day 20 after DSS treatment.

PPA, Pulsatilla Koreana NAKAI pharmacopuncture.

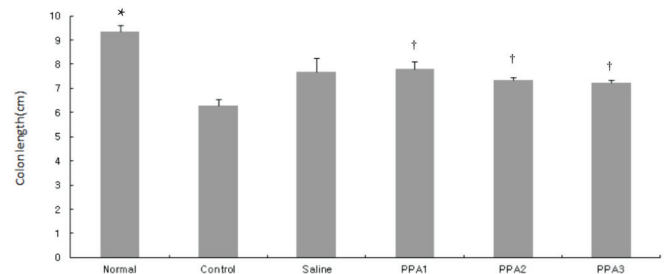


Fig. 3. Effect of pharmacopuncture (Pulsatilla koreana, Nakai) at ST25 on colon lengths in DSS-induced colitis.

**p* <0.001,
 †*p* <0.05 compared with control.
 DSS, Dextran sulfate sodium; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST25, Chunchu.

Effect on the mucosal tissues of colon

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on the mucosal tissues of colon, In comparison with the normal group, control group's mucosal layer disappeared in the upper part of the colon mucosa and fibrosis progressed with inflammation and congestive reaction. In the lower part, mucosal layer disappeared due to inflammation as well as lymphocytic infiltration in the basal layer.

In all groups treated with Pulsatilla pharmacopuncture therapy compared to the control group, the disappearance of the upper and

lower mucosal layer was reduced by the relief of inflammation and congestive reaction. The lymphocyte infiltration of the lower basal layer was also found to disappear close to the normal group (Fig. 4).

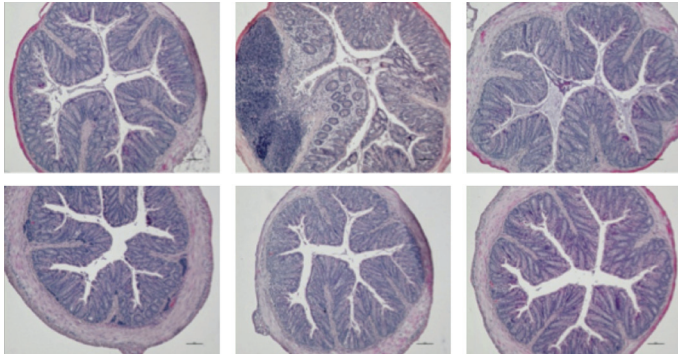


Fig. 4. Representative microphotographs of coronal sections of colonic mucosa from a mice.
H-E stain (magnification 100×).

Effect on weight change

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on weight change, In the control group, 21.0 ± 0.43 g before treatment, 23.0 ± 0.22 g at the first treatment, 22.8 ± 0.37 g at the second treatment, 21.8 ± 0.00 g at the third treatment, 23.2 ± 0.22 g at the fourth treatment, and 23.4 ± 0.26 g at the fifth treatment, and 23.4 ± 0.26 g at 6th treatment, respectively, showing a tendency to decrease compared with the normal group, especially in the third of Pulsatilla pharmacopuncture therapy. The PPA1 ($p < 0.05$) and PPA2 ($p < 0.05$) groups were significantly increased in the second. Saline ($p < 0.001$), PPA1 ($p < 0.001$), PPA2 ($p < 0.001$) and PPA3 ($p < 0.001$) groups were significantly increased in the third. PPA1 ($p < 0.05$) group was significantly increased in the sixth (Table 4, Fig. 5).

Effect on IL-6

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change of IL-6, 86.6 ± 1.65 (* 1,000 OD) in the normal group, 123.3 ± 3.83 (* 1,000 OD) in the control

Table 4. Changes on the Body Weight.

Group	0	Pharmacopuncture					
		1	2	3	4	5	6
Normal(g)	21.2±0.20	23.0±0.63	23.6±0.51	23.6±0.51*	23.8±0.73	24.0±0.84	24.8±0.73
Control(g)	21.0±0.43	23.0±0.22	22.8±0.37	21.8±0.00	23.2±0.22	23.4±0.26	23.4±0.26
Saline(g)	21.6±0.24	23.2±0.37	24.0±0.55	24.4±0.40‡	24.2±0.37	24.2±0.58	24.2±0.58
PPA1(g)	21.6±0.24	23.4±0.40	24.2±0.37†	24.4±0.40‡	24.0±0.32	23.8±0.20	24.4±0.24†
PPA2(g)	21.8±0.20	23.6±0.24	23.8±0.20†	24.2±0.20‡	24.0±0.32	24.0±0.32	24.0±0.32
PPA3(g)	21.2±0.20	22.0±0.55	22.6±0.40	23.2±0.49‡	22.6±0.51	22.6±0.51	23.0±0.63

Values are presented as mean ± SE.

* $p < 0.05$.

† $p < 0.05$ compared with control.

‡ $p < 0.001$ compared with control

PPA, Pulsatilla Koreana NAKAI pharmacopuncture.

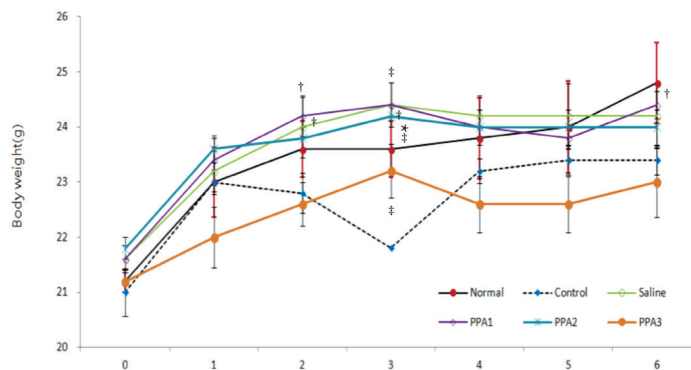


Fig 5. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on body weight in DSS-induced colitis.

Values are presented as mean ± SE.

* $p < 0.05$

† $p < 0.05$ compared with control.

‡ $p < 0.001$ compared with control.

group and 99.6 ± 2.74 (* 1,000 OD) in Saline group, 94.8 ± 2.60 (* 1,000 OD) in the PPA1 group, 79.1 ± 1.36 (* 1,000 OD) in the PPA2 group and 84.9 ± 2.09 (* 1,000 OD) in the PPA3 group, respectively.

Compared with the control group, saline group ($p < 0.05$), PPA1 ($p < 0.05$), PPA2 ($p < 0.001$), and PPA3 ($p < 0.001$) groups showed a significant decrease (Fig. 6).

Effect on IL-10

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change of IL-10, 113.7 ± 2.33 (*1,000 O.D) in the normal group, 101.1 ± 0.40 (*1,000 O.D) in the control group, 114.6 ± 1.65 (*1,000 O.D) in Saline group, 99.4 ± 1.13 (*1,000 O.D) in the PPA1 group, 95.0 ± 1.03 (*1,000 O.D) in the PPA2 group and 114.6 ± 2.06 (*1,000 O.D) in the PPA3 group, respectively. Significant increases were noted in the saline group and the PPA3 group, compared to the control group ($p < 0.05$). There was a significant decrease in the PPA2 group, compared to the control group ($p < 0.05$) (Fig. 7).

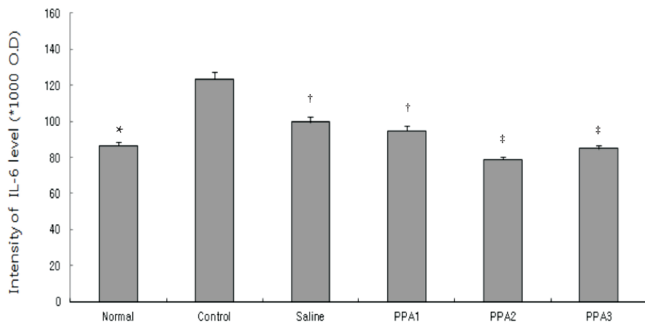


Fig 6. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of IL-6 in DSS-induced colitis.

Values are presented as mean \pm SE.

* $p < 0.001$

† $p < 0.05$

‡ $p < 0.001$ compared with control

IL, interleukin; PPA, Pulsatilla Koreana NAKAI pharmacopuncture.

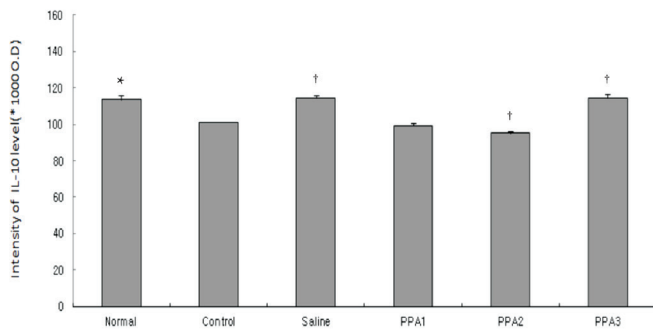


Fig 7. Effect of Pharmacopuncture(Pulsatilla koreana Nakai) at ST25 on Level of IL-10 in DSS-induced Colitis.

Values are presented as mean \pm SE.

* $p < 0.05$

† $p < 0.05$ compared with control

DSS, Dextran sulfate sodium; IL, interleukin; ST, Chunchu.

Effect on IL-1 β

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change of IL-1 β , 66.3 ± 4.69 (*1,000 O.D) in the normal group, 89.7 ± 2.38 (*1,000 O.D) in the control group, 79.4 ± 2.18 (*1,000 O.D) in Saline group, 85.4 ± 1.82 (*1,000 O.D) in the PPA1 group, 61.2 ± 2.76 (*1,000 O.D) in the PPA2 group and 81.3 ± 2.44 (*1,000 O.D) in the PPA3 group, respectively. Compared with the control group, there was a significant decrease in the saline group and the PPA2 group ($p < 0.05$) (Fig. 8).

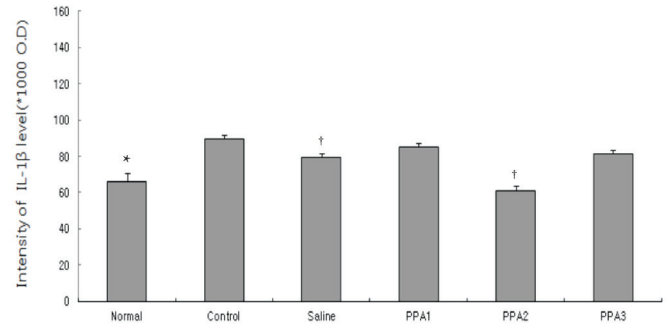


Fig 8. Effect of Pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on Level of IL-1 β in DSS-induced Colitis.

Values are presented as mean \pm SE.

* $p < 0.05$,

† $p < 0.05$ compared with control.

DSS, Dextran sulfate sodium; IL, interleukin; ST, Chunchu.

Effect on Interferon- γ (IFN- γ)

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change IFN- γ , 58.8 ± 1.43 (*1,000 O.D) in the normal group, 138.9 ± 1.05 (*1,000 O.D) in the control group, 140.2 ± 1.45 (*1,000 O.D) in Saline group, 137.2 ± 1.76 (*1,000 O.D) in the PPA1 group, 91.9 ± 1.41 (*1,000 O.D) in the PPA2 group and 126.3 ± 1.07 (*1,000 O.D) in the PPA3 group, respectively. Compared with the control group, there was a significant decrease in the PPA2 group ($p < 0.001$) and the PPA3 group ($p < 0.05$)(Fig. 9).

Effect on tumor necrosis factor- α (TNF- α)

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change TNF- α , 85.7 ± 3.52 (*1,000 O.D) in the normal group, 129.0 ± 4.29 (*1,000 O.D) in the control group, 128.5 ± 4.24 (*1,000 O.D) in Saline group, 43.8 ± 1.90 (*1,000 O.D) in the PPA1 group, 69.8 ± 1.88 (*1,000 O.D) in the PPA2 group and 101.0 ± 2.40 (*1,000 O.D) in the PPA3 group, respectively. Compared with the control group, there was a significant decrease in the PPA1 group ($p < 0.001$), the PPA2 group ($p < 0.001$), and the PPA3 group ($p < 0.05$) (Fig. 10).

Effect on transforming growth factor- β 1 (TGF- β 1)

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change TGF- β 1, 121.8 ± 3.23 (*1,000 O.D) in the normal group, 105.5 ± 4.24 (*1,000 O.D) in the control group, 118.5 ± 3.12 (*1,000 O.D) in Saline group,

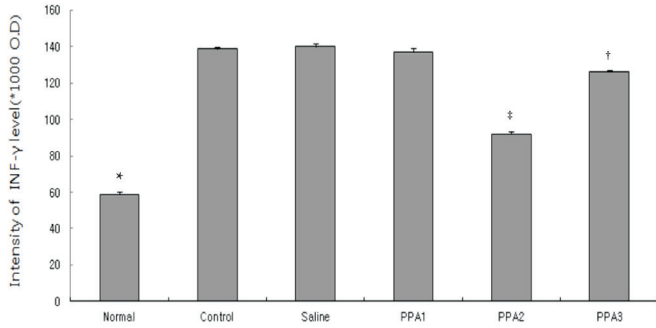


Fig. 9. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of IFN- γ in DSS-induced colitis.

Values are presented as mean \pm SE.

* $p < 0.001$

† $p < 0.05$

‡ $p < 0.001$, compared with control

DSS, Dextran sulfate sodium; INF, interferon; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST, Chunchu.

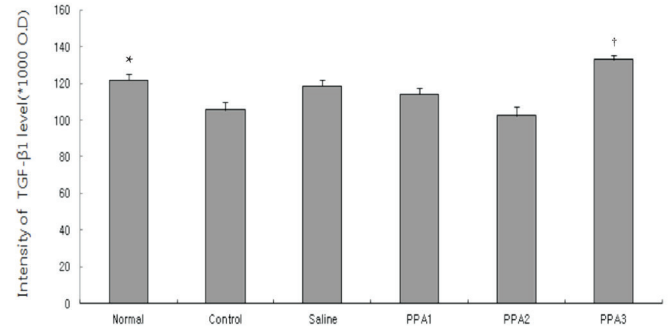


Fig. 11. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of TGF- β 1 in DSS-induced colitis.

Values are presented as mean \pm SE.

* $p < 0.001$

† $p < 0.05$, compared with control

DSS, Dextran sulfate sodium; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST, Chunchu; TGF, tumor necrosis factor.

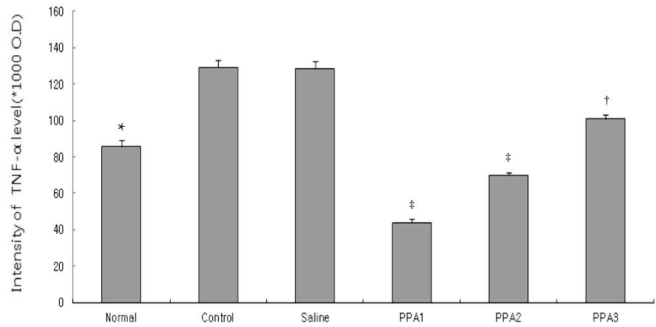


Fig. 10. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of TNF- α in DSS-induced colitis.

Values are presented as mean \pm SE.

* $p < 0.05$,

† $p < 0.05$, compared with control

‡ $p < 0.001$, compared with control.

DSS, Dextran sulfate sodium; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST, Chunchu; TNE, transforming growth factor.

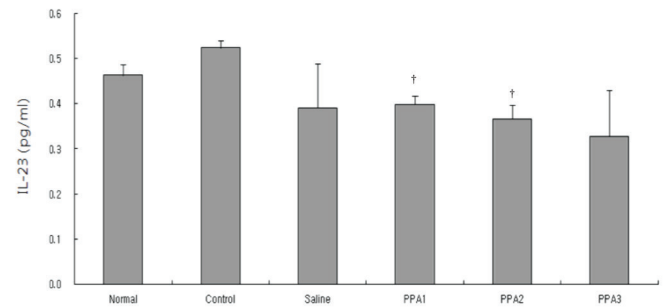


Fig. 12. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of IL-23 in DSS-induced colitis.

Values are presented as mean \pm SE.

* $p < 0.05$, compared with control

DSS, Dextran sulfate sodium; IL, interleukin; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST, Chunchu.

114.1 \pm 3.41 (*1,000 O.D) in the PPA1 group, 102.4 \pm 5.16 (*1,000 O.D) in the PPA2 group and 132.9 \pm 2.48 (*1,000 O.D) in the PPA3 group, respectively. Compared with the control group, there was a significant increase in the PPA3 group ($p < 0.05$) (Fig. 11).

Effect on IL-23

As a result of observing the effect of Pulsatilla pharmacopuncture therapy at Chunchu (ST25) on change IL-23, 0.46 \pm 0.023 pg/ml in the normal group, 0.52 \pm 0.017 pg/ml in the control group, 0.39 \pm 0.098 pg/ml in Saline group, 0.40 \pm 0.018 pg/ml in the PPA1 group, 0.37 \pm 0.030 pg/ml in the PPA2 group and 0.33 \pm 0.102 pg/ml in the PPA3 group, respectively. Compared with the control group, there was a significant decrease in the PPA1 group, and the PPA2 group ($p < 0.05$) (Fig. 12).

Effect on IL-17

As a result of observing the effect of Pulsatilla pharmacopuncture

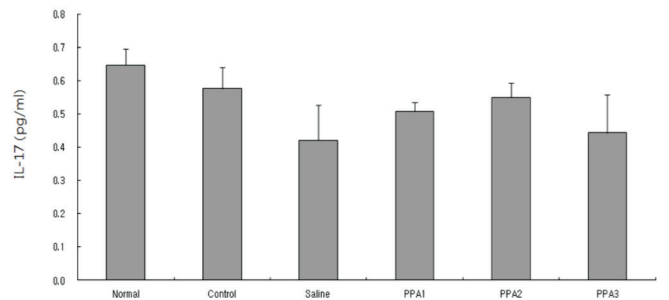


Fig. 13. Effect of pharmacopuncture (Pulsatilla koreana Nakai) at ST25 on level of IL-17 in DSS-induced colitis.

Values are presented as mean \pm SE.

DSS, Dextran sulfate sodium; IL, interleukin; PPA, Pulsatilla Koreana NAKAI pharmacopuncture; ST, Chunchu.

therapy at Chunchu (ST25) on change IL-17, 0.65 ± 0.050 pg/ml in the normal group, 0.58 ± 0.063 pg/ml in the control group, 0.42 ± 0.106 pg/ml in Saline group, 0.51 ± 0.026 pg/ml in the PPA1 group, 0.55 ± 0.043 pg/ml in the PPA2 group and 0.44 ± 0.114 pg/ml in the PPA3 group, respectively. Compared with the control group, did not show any significant change in all experimental groups (Fig. 13).

Discussion

Colitis, which is divided into ulcerative colitis and Crohn's disease, is a chronic inflammatory disease of the unknown origin, which in a broad sense includes inflammatory bowel diseases such as bacterial enteritis, ischemic enteritis, amoebic dysentery, Behcet's enteritis and tuberculous enteritis [14].

Ulcerative colitis is a disease that causes symptoms such as abdominal pain, hemorrhage, diarrhea, fever and weight loss by invading the mucous membrane and submucosal layer of the rectum. There is a history of clinically repeated hemorrhagic diarrhea or occult blood [15]. Crohn's disease which can invade the entire gastrointestinal tract is a chronic inflammatory disease of the small intestine, distal end of the ileum, and colon. Unlike ulcerative colitis, abdominal pain, weight loss, and fistula around the anus are more common than hemorrhagic diarrhea [16].

The causes of colitis which have not been clearly elucidated are presumed to be environmental factors such as smoking, dietary factors, genetic factors such as immune response due to exposure of inducers to people with genetic predisposition [17], immune system abnormalities due to imbalance of intestinal microorganisms [14], and bacterial infection. Colitis rarely occurred in Asia, South America and Africa, but the prevalence of Asia is on the rise.

Since the cause and pathophysiology of the colitis is unclear, the basic pharmacotherapy system is insufficient. Therefore, it is aimed to maintain and improve the symptom level to prevent serious complications with conservative and symptomatic treatment such as sap, nutritional supplementation [19]. In case of complications such as colon perforation, no response to medical treatment, and colorectal cancer, surgical operation is performed [20]. Medicines that are used for the treatment of symptomatic treatment are steroid preparations and aminosalicylate drugs such as sulfasalazine and mesalazine. When those are not responsive to such drug, Immunosuppressive drugs such as azathioprine, 6-mercaptopurine, and cyclosporin are also used, but they can not be expected to be completely cured and have a high recurrence rate after relieving symptoms. Long-term administration is associated with anorexia, dyspepsia, hepatitis, hemolytic anemia and other side effects and resistance to drugs may also occur [21].

Many studies on colitis have been reported, including Korean medicine, prescription of single herb, acupuncture and pharmacopuncture. Depending on the drugs that cause colitis, different treatment methods are dominant. Studies of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis were more frequently performed with pharmacopuncture such as *Rhizoma coptidis* [24], *Ginseng radix* [25], and *Artemisia argyi* [26]. Studies of DSS-induced colitis have been carried out using Korean medicine such as *Soyumjungjang-tang* [5], *Jiyutang* [6], *Doche-tang* [27], *Bokijeongjang-tang* [28], *Hwangryunhaedoktang* [7], using prescription of single herb such as *Aucklandia lappa* [8], *Mume Fructus* [9], *Adenophorae Radix* [29], *Scutellariae Radix* [30], *Orostachys japonica* [10]. Overall, there were a number of studies using Korean medicine and prescription of single herb, few studies using pharmacopuncture, and very few studies using acupuncture alone.

In this study, DSS, which is used to induce colitis, is a polyanionic compound containing three sulfate groups depending on the glucose molecular weight. The oral administration of DSS causes phagocytosis of the macrophages, which changes the composition of the serosa and stimulates the luminal antigen inflow into the mucosa, resulting in intestinal inflammation [31]. Inducing colitis by DSS which is oral administration is less likely to induce experimental errors than injecting TNBS into the anus via [7]. It is characterized by mucosal invasion, epithelial cell damage, and ulceration, similar to human ulcerative colitis appears [32].

Pulsatilla (*Radix Pulsatilla koreana* Nakai), used as a pharmacopuncture solution, is a dried root of *Pulsatilla koreana* NAKAI or related species in the same genus, perennial herb that belongs to Ranunculaceae. It is distributed in Korea and the northeast of China and collected in spring and autumn. It has the effect of clearing away heat and eliminating toxin and cooling blood and stopping diarrhea with bitter and cold taste. It contains components such as anemonin and pulsatoside. When hydrolyzed, it produces sapogenin ST-1, glucose and shows a powerful inhibitory action against amoebae, shigella and staphylococcus aureus [33]. In previous studies, Park et al [34] identified the anti-inflammatory effect of *Radix Pulsatilla koreana* Nakai extract and Cho et al [35] demonstrated its antioxidative effect.

Chunchu (ST25), used as the injection point, which is front point of colon is taken from the left and right side of the navel 6cm. It has the effect of communicating and control the colon, harmonize the stomach, reinforcing earth and remove moisture. Therefore enteritis, diarrhea, dysentery can be treated [36]. This is the basis for the Chunchu (ST25) to treat the colonic disease.

The purpose of this study was to investigate the treatment methods of colitis in Korean medicine by using pharmacopuncture which is based on the reports of previous studies. The study was conducted through DSS to induce colitis in the mice and to perform pharmacopuncture at Chunchu (ST25), and then to measure the DAI, colon length and mucosal tissue, body weight change, gene expression and cytokine concentration.

DAI which is comprehensive functional measures similar to clinical signs of ulcerative colitis, examines three major signs of weight loss, diarrhea and rectal bleeding. Weight loss is defined as the difference between the initial body weight and the current body weight. Diarrhea is defined as persistent soft excreta in the rectum, and rectal bleeding is defined as hemorrhagic diarrhea and total rectal bleeding [37].

As a result of observing changes of DAI in this experiment, the control group was significantly increased compared to the normal group, during the period of up to 6th treatment after DSS - induced colitis. Compared with the control group, the PPA1 group decreased significantly in the second, all PPA groups decreased significantly in the fourth and sixth.

The changes in the length of the colon were significantly increased in all PPA groups compared to the control group. Changes in body weight tend to decrease in the control group compared to the normal group, significantly decreased third. PPA1 and PPA2 groups were significantly increased in the second, saline group and all the PPA groups were significantly increased in the third, PPA1 group was significantly increased in the sixth.

In the study of Yang et al [12], which applied the *Sophorae Radix* Pharmacopuncture Therapy to Chunchu (ST25), and the study of Lee et al [38], which applied the *Hwangryeonhaedoktang* Pharmacopuncture to Chunchu (ST25), the changes in length and weight of the colon were significant, and it was judged that Chunchu (ST25) itself had a stimulating effect as well as the effect of the pharmacopuncture itself.

Changes in the mucosal tissues of the colon showed that

all the PPA groups had less inflammatory and congestive responses compared to the control group with mucosal layer loss, inflammation and congestive response, fibrosis, and lymphocyte infiltration of the basal layer. Mucosal layer loss, and basal layer lymphocyte infiltration were observed to close to normal group. Thus, we could confirm the effect of inhibiting tissue deformation and inflammation by Pulsatilla pharmacopuncture therapy at Chunchu (ST25).

Cytokine is an important activator of immune system regulation. It is activated in most immune, inflammatory and infectious diseases. It induces inflammation, deepens inflammation, relieves inflammation. Imbalance of inflammatory cytokine and anti-inflammatory cytokine is found in colitis [39].

Among cytokines related to immunity and inflammation, IL-6, TNF- α , and IL-1 β are inflammatory cytokines produced by macrophages. IL-6 plays a central role in immune regulation and inflammation, and is significantly increased in the case of colitis. The secretion of TNF- α , which is present at a high concentration in inflammation site, synthesizes mediators such as prostaglandins and NO to induce tissue damage and induce an inflammatory reaction [40]. IL-1 β acts on endothelial cells and epithelial cells to induce inflammation and hematopoiesis, while IFN- γ induces other cytokines involved in the inflammatory process when expressed as an immunomodulatory [41].

In the present study, IL-6 was significantly decreased in the saline group, PPA1 group, PPA2 group and PPA3 group compared to the control group. IL-1 β was significantly decreased in the saline group and the PPA2 group. IFN- γ was significantly decreased in the PPA2 and PPA3 groups. TNF- α was significantly decreased in the PPA1, PPA2, and PPA3 groups.

The expression of inflammatory cytokine was significantly decreased in most groups, suggesting that the inflammatory response was suppressed. Especially, PPA2 group was significantly decreased in all four cytokines, and it is considered to be most effective in suppressing inflammation.

IL-10 and TGF- β , which are anti-inflammatory cytokines, affect the inflammatory capacity of Tregs, which are considered to be important mechanisms of colitis. IL-10 promotes Treg differentiation, inhibits the production of IL-6 and TNF- α , modulates the inflammatory response [42]. And then TGF- β acts as a suppressive cytokine, a major regulator of immune homeostasis and inflammatory response [43].

In the present study, IL-10 was significantly increased in the saline group and PPA3 group and significantly decreased in the PPA2 group compared to the control group, and the TGF- β 1 was significantly increased in the PPA3 group.

The level of anti-inflammatory cytokine expression was significantly increased in most groups. Especially, PPA3 group showed the highest anti-inflammatory effect in both cytokines. In addition, the PPA2 group, which was judged to be effective in suppressing inflammation with a significant decrease in all inflammatory cytokines showed a significant decrease in IL-10. Therefore, PPA2 inhibited inflammatory cytokines but did not induce the activity of anti-inflammatory cytokines.

The inflammatory mechanism of colitis is associated with Th1 and Th17, so treatment is also required to inhibit both [44]. In particular, Th17, which secretes IL-17, is known to play an important role in the development and activity of immune system diseases, and IL-23, which directly activates these cells, is also receiving attention. It is known that L-23 is involved in the survival and expansion of Th17 and increases IL-17 expression [45]. IL-17 is also involved in the expression of IL-23 at the time of Th17 differentiation [46].

In the present study, the changes of IL-23 concentration were

significantly decreased in the PPA1 and PPA2 groups compared to the control group, and all the experimental groups did not show a significant change in the concentration of IL-17.

Because IL-23 and IL-17 did not show the same direction as the increase or decrease, the interaction between two cytokines could not be confirmed. Also the therapeutic effect of PPA could not be observed.

From the above results, DAI score was decreased because of preservation of colon length and preservation of body weight by suppressing deformation of colon mucosal tissue and relieving inflammation, and significant decrease of inflammatory cytokine and significant increase of anti-inflammatory cytokine Chunchu (ST25) was found to be an effective treatment for inflammatory bowel disease induced inflammatory bowel disease including colitis. There was a significant decrease in inflammatory cytokine and a significant increase in anti-inflammatory cytokine, indicating that Pulsatilla pharmacopuncture therapy at Chunchu (ST25) could be an effective treatment for inducing inflammation-suppressing response in inflammatory bowel disease including colitis.

However, in order to confirm the significance between the concentrations, it is necessary to verify the difference of immunity mechanism between the PPA2 group which was effective for the inhibition of the inflammatory cytokine and the PPA3 group which was effective for the activity of the anti-inflammatory cytokine.

In addition, a complementary study to confirm interaction of IL-17 and IL-23 cytokines and the relationship of IL-17 and IL-23 cytokines to PPA, which did not yield significant results, is also needed.

Conclusion

In order to experimentally evaluate the effect of Pulsatilla pharmacopuncture therapy on colitis, we performed Chunchu (ST25)-Pulsatilla pharmacopuncture therapy on Dextran sulfate sodium-induced colitis mice and observed changes in DAI, colon length, mucosal tissues and body weight. In addition gene expression by RT-PCR and Cytokine concentration were measured and the following results were obtained.

- 1) DAI was significantly decreased in the PPA1 group at second and in all PPA groups at fourth and sixth compared to the control group.
- 2) The length of the colon was significantly increased in Saline and all PPA groups compared to the control group
- 3) Histological findings of colonic mucosal tissues showed that all the PPA groups showed less inflammation and congestive responses than the control group.
- 4) Body weight was significantly increased in the PPA1 and PPA2 groups in the second, Saline and all PPA groups in the third, and PPA1 in the sixth compared to the control group.
- 5) The PPA2 group showed a significant decrease in IL-6, IL-1 β , IFN- γ and TNF- α expression levels and IL-23 concentration compared to the control group.
- 6) The expression of IL-10 and TGF- β 1 in the PPA3 group was significantly increased compared to the control group.
- 7) IL-17 levels were not significantly changed in all experimental groups compared to the control group.

Based on the above results, Pulsatilla pharmacopuncture therapy at Chunchu (ST25) could be used as an effective treatment for inflammatory bowel disease through inhibition of intestinal mucosal tissue damage and reduction of inflammatory mediator production.

Conflicts of interest

The authors have no conflicts of interest to declare.

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