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Role of Sishen pill in treating inflammatory bowel diseases through regulating the metabolism of memory Treg cells: An exploration based on the theory of benefiting source of fire and eliminating yin

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Abstract: The incidence of inflammatory bowel diseases (IBDs) is increasing yearly and treatment options remain limited. Sishen Pill (SSP), a Chinese medicine, may aid IBD by impacting energy metabolism and immune response in memory Treg cells, though its exact mechanism remains unclear. This study was designed to investigate the SSP's mechanism in IBD treatment via regulating memory T cells based on the theory of Benefiting Source of Fire and Eliminating Yin. A spleen-kidney yang deficiency model was induced in mice using rhubarb decoction and hydrocortisone, followed by a DSS-induced IBD model. Mice were treated with SSP at varying doses. Disease activity index (DAI) scores, colonic weight index and histological injury scores were measured. Flow cytometry quantified T cell subsets and ELISA tests assessed TNF-α, IL-17 and energy metabolites (ATP, ADP and AMP). The establishment of an ulcerative colitis mouse model with spleen-kidney yang deficiency was conducted. As opposed to controls, DSS increased TEM (CCR7-CD45RA-) and Foxp3+ T-cell ratios and reduced TCM (CCR7+CD45RA-) (P<0.05). SSP dose-dependently improved colitis indicators, decreased TNF-α, IL-17 and enhanced energy metabolism by modulating ATP, ADP and AMP levels (P<0.05). SSP may alleviate DSS-induced IBDs by regulating memory T cell metabolism and energy metabolism.

Keywords: Sishen pill (SSP), inflammatory bowel diseases (IBDs), memory Treg (T) cell, dextran sulfate sodium (DSS), energy metabolism

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic condition marked by recurrent intestinal inflammation. As reported, roughly 25% IBD patients turn into colorectal cancer (Siegel *et al.,* 2019). In China, the prevalence of IBD has steadily increased, with rates reaching over 3 cases per 100,000 annually. Worldwide, IBD affects more than 6.8 million people, particularly in Western countries where prevalence rates are highest (Kaplan and Ng, 2016). As a recognized precancerous lesion, IBD, including ulcerative colitis and Crohn's disease, mainly refers to pathological features such as inflammatory response and ulcer due to the intestinal mucosal barrier damage induced by a variety of factors. Despite increasing incidence and recurrence year by year (Chang, 2020), the specific pathogenesis of IBD remains to be clarified. Currently, IBD has been proven to be related to energy metabolism disorder, sustained immune response, intestinal flora change, genetic susceptibility and environmental factors (Wang *et al.,* 2022). Sustained immune response refers to the specific immune reaction generated by the body's immune system following exposure to an antigen.

Generally, upon impact of the same antigen, the body will produce a more significant sustained immune response. As stated by numerous studies, this abnormal immune response is closely related to the pro-inflammatory memory Treg (T) cells in the bowel of IBD patients. For instance, patients with intestinal inflammations are prone to be recurrent rather than to be cured and alleviated, due to sustained immune response (Brincks *et al.,* 2013). Hence, regulating inflammatory memory T cells may be an effective therapeutic regimen to inhibit the malignant development of IBD. In addition, the disorder of cell energy metabolism is also one of the essential characteristics of IBD. As important parts of colon barriers, the cytoskeletons require sufficient adenosine triphosphate (ATP) so that enough energy can be provided to maintain tension (Buelto and Duncan, 2014). Therefore, the therapeutic drugs targeting sustained immune response and energy metabolism disorder contribute to alleviating IBD.

Sishen Pill (SSP) is a Chinese patent medicine consisting of *Evodia rutaecarpa, Psoralea corylifolia* Linn*., Schisandra chinensis*, nutmeg, ginger and jujube. According to the traditional Chinese medicine theory, SSP plays a role in benefiting sources of fire and eliminating yin, which means that it has the functions of clearing away heat and detoxication, inhibiting inflammation and

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nourishing yin. Hence, SSP is considered to be an effective drug for treating IBD (Liu *et al.,* 2020). As reported by related literature, the fundamental mechanism of SSP is to regulate the cell energy metabolism and balance the cell immune system. Moreover, the function of benefiting source of fire and eliminating yin may help to regulate the human immune system, alleviate inflammatory responses and promote the repair of intestinal tissue (Chen *et al.,* 2020). The TCM theory of "benefiting the source of fire and eliminating yin" traditionally refers to promoting vitality and reducing pathological dampness or stagnation. Modern interpretations correlate these concepts with enhancing cellular energy metabolism and controlling inflammatory responses. SSP, through its impact on ATP, ADP and AMP in memory Treg cells, supports energy homeostasis, reflecting the "source of fire" concept (Wang *et al.,* 2022), while its reduction of TNF-α and IL-17 levels aligns with the "eliminating yin" approach by decreasing inflammatory responses (Chen *et al.,* 2020).

This study investigated the SSP's potential to bridge these TCM principles with cellular metabolism and immune regulation. However, it is not clear whether SSP alleviates IBD by modulating the memory T cells' energy metabolism and immune response in colon in accordance with the theory of Benefiting Source of Fire and Eliminating Yin up to now. Therefore, we put forward the hypothesis that SSP alleviated IBD by regulating the memory T cells' energy metabolism and immune response in the colon according to this theory. After verification of such a hypothesis, this study provided a vital theoretical basis for applying SSP in IBD treatment.

MATERIALS AND METHODS

Materials

Experimental drugs: SSP (the batch number: 17080051) was purchased from Beijing Tongrentang Natural Medicines Co., Ltd. (China). Experimental animals: Fifty C57BL/6J male mice (WT) aged 9-12 weeks were provided by Hunan SJA Laboratory Animal Co., Ltd.

Animal modeling, grouping and administration intervention

This study was conducted at the College of Traditional Chinese Medicine, Nanchang Medical College and adhered to guidelines approved by the college's Ethics Committee. The spleen-kidney yang deficiency model was induced by rhubarb decoction combined with hydrocortisone, the model of the IBD mouse was established using dextran sulfate sodium (DSS) and finally, a model incorporating both ulcerative colitis mouse and kidney-yang deficiency in mice was constructed (Vlantis *et al.,* 2016). Fig. 1 presented the detailed modeling and grouping. Briefly, 1 ml rhubarb decoction (250mg/ml) was gavaged for 7 days and on the 8th day, 25mg/(kg.d) hydrocortisone was alternately injected into the left and right hip muscles daily for 7 days. Besides, 2% DSS solution was prepared with normal drinking water and the fecal occult blood test was carried out based on the Fecal OB-II method to check whether the modeling was successful. If the modeling was still unsuccessful on the 15th day, the application of 2% DSS was discontinued and drinking water was provided only for 5 days. On the 20th day, mice had free access to drinking 1.5% DSS solution for 7 consecutive days until the modeling was successful.

SSP was administered at three dosage levels: 1.25g/kg (low), 5g/kg (high) and 2.5 g/kg (medium) body weight, where 5g/kg maximized observable immune-modulatory benefits, 1.25g/kg served as the minimal effective dose, and 2.5g/kg provided a medium range for comparison. This selection aimed to delineate a dose-response relationship and assessed SSP's therapeutic threshold (Huang *et al.,* 2007). Treatments were delivered orally once daily over a period of seven days following successful modeling. Mice in the DSS-only group received a saline solution at 300 mg/kg for comparison.

Subsequently, 50 mice were allocated into five groups at random (n=10): The control, DSS, DSS+SSP-L, DSS+SSP-M and DSS+SSP-H groups. Aside from being fed normally, the control group mice received no other treatments. Random assignment of the remaining 40 mice was made to the DSS group, DSS+SSP-L group, DSS+SSP-M group and DSS+SSP-H group and subjected to establishing IBD mouse models with a spleen-kidney yang deficiency pattern according to the above methods. On the 15th day, the medicine intervention was launched. Mice in the DSS+SSP-L group, DSS+SSP-M group, and DSS+SSP-H group were separately given different doses of SSP (medium, 2.5 g/kg; high, 5 g/kg; low, 1.25 g/kg). The DSS group received 300 mg/kg normal saline. The intervention involved daily administration of the medicine for 7 days. From the date of medicine intervention to before the animals were killed, daily observation and evaluation of the disease activity index (DAI) were conducted, including the body weight, fecal viscosity and hematochezia. On the 29th day, before and after the mice were killed, the cringe, emaciation, colon thickening and fecal characteristics were observed, and the clinical score of colitis was calculated. In addition, on the final day of treatment, the mice underwent a 12-h fast, deep anesthesia with 3% pentobarbital sodium and were then euthanized. Next, the colon tissue was separated and the colonic weight was measured. Finally, the colonic weight index was calculated according to the formula of colonic weight index = colon mass (g)/animal weight (g)×100%.

Preparation of pathological section of colon and pathological scoring criteria

The colon tissues underwent fixation with 4% paraformaldehyde, dehydration, paraffin embedding and

sectioning. Upon staining with hematoxylin for 1 min, the sections were subjected to rinsing with tap water. Next, the differentiation process involved a few seconds of ethyl alcohol containing 1% hydrochloric acid, followed by a tap water rinse and 1 minute of bluing in 1% ammonia. Following a rinse in running water, the sections were subjected to several seconds of eosin staining, followed by a second rinse with running water. Subsequently, immersion of the sections was conducted in 75% ethanol (2 min), 85% ethanol (2 min) and anhydrous ethanol (5 min), with a repeated immersion in anhydrous ethanol for another 5 min. The sections underwent 5 min of transparency using xylene, after which they were removed and sealed using neutral gum. Next, a microscope was applied for observing the colon tissue's pathological changes. Lastly, the colon's pathological damage under the histopathology was scored.

Flow cytometry

The T cell subset levels were tested via flow cytometry. Briefly, the mouse colon tissue was placed on an ultra-clean table to remove the surrounding connective tissue. After adding an appropriate amount of PBS, the colon tissues were fully ground and filtered to prepare a single cell suspension. Then, resuspension of the cells was conducted in 100μL medium with fetal bovine serum (FBS). Upon membrane fixation and permeabilization (Vlantis *et al.,* 2016), the cells underwent washes with buffer solution, followed by staining with anti-CC-chemokine receptor 7 (CCR7, ab191575, 1:200), anti-CD45RA (MEM-56, 1:200) and anti-FOXP3 (ab75763) monoclonal antibodies at 4^{o}C for 30 min. Subsequently, the percentages of central memory T cell (TCM, CCR7+CD45RA-) and effector memory T cell (TEM, CCR7-CD45RA-) subsets were determined within 1 h using a Moflo^{TM} cell sorter (Beckman Coulter), and cells of two subsets were selected. Later, the percentage of FOXP3+ cells in the two subsets was detected, respectively. Finally, FlowJo 7.6.1 software was applied for analyzing and processing the flow cytometry outcomes.

ELISA

Assessment was conducted on the interleukin ATP, (IL)-17, tumor necrosis factor alpha (TNF-α), adenosine monophosphate (AMP) and adenosine diphosphate (ADP) levels using ELISA. Firstly, the mouse colon tissues were cut into pieces, lysed with lysate for 1h at ambient temperature, and then homogenized. The homogenate was centrifuged at 4^{o}C and 30 min later, collection of the supernatant was performed. Then, TNF-α (JYM0635Ra), IL-17 (JYM0554Mo), ATP (JYM0723Mo), ADP (JYM0725Mo) and AMP (JYM0721Mo) contents in colon tissue homogenate were detected as per the instructions of commercial kits (Wuhan ColorfulGene Biological Technology); and the absorbance value was determined at 450nm.

STATISTICAL ANALYSIS

Mean ± standard deviation (SD) was adopted for presenting the data results. Statistical analysis was conducted via SPSS 24.0 and Graphpad Prism 9.0. A one-way ANOVA was applied for comparing the data among multiple groups. $P<0.05$ represented the statistical significance threshold.

Ethical approval

This study was approved by the Ethics Committee of Nanchang Medical College (NCMC-2024-3618) and conducted in accordance with the approval guidelines. The study was carried out in compliance with the ARRIVE guidelines.

RESULTS

Efficacy of Sishen Pill in treating ulcerative colitis mice with a spleen-kidney yang deficiency pattern

A mouse model with ulcerative colitis and spleen-kidney yang deficiency was constructed for investigating the SSP's therapeutic effect on ulcerative colitis with spleen-kidney yang deficiency in mice. Also, three SSP doses (5 g/kg, 2.5g/kg and 1.25g/kg) were administered. After administration, the DAI, clinical score of colitis, histological injury score and colonic weight index of mice in each group were displayed in fig. 2. As opposed to the control group, the DAI, clinical score of colitis, colonic histological injury score and colonic weight index were markedly raised in the DSS group ($P<0.05$), with more severe inflammatory cell infiltration. In comparison with the DSS group, a notable decline was observed in DAI, histological injury score, clinical score of colitis, and colonic weight index in the DSS+SSP-L, DSS+SSP-M and DSS+SSP-H groups ($P<0.05$). SSP treatment alleviated inflammatory cell infiltration and histological injury in a dose-dependent manner, with higher SSP doses indicating a lower scoring level and a better improvement effect. The aforementioned outcomes suggested that SSP was effective in improving ulcerative colitis mice with a spleen-kidney yang deficiency pattern.

Effect of Sishen Pill on immunological memory Treg cell subsets and Foxp3+ cells in ulcerative colitis mice with a spleen-kidney yang deficiency type

Further, we determined the SSP's effect on immunological memory T cell subsets and Foxp3+ cells in ulcerative colitis mice with a spleen-kidney yang deficiency type. As confirmed by the outcomes, relative to the control group the DSS group exhibited a remarkable reduction in the TCM (CCR7+CD45RA-) ratio in mouse colon tissues ($P<0.01$), accompanied by a notable raise in TEM (CCR7-CD45RA-), CCR7-CD45RA-Foxp3+T and CCR7+CD45RA-Foxp3+ ratios ($P<0.05$). As opposed to the DSS group, the DSS+SSP-L, DSS+SSP-M and DSS+SSP-H groups exhibited notably raised TCM

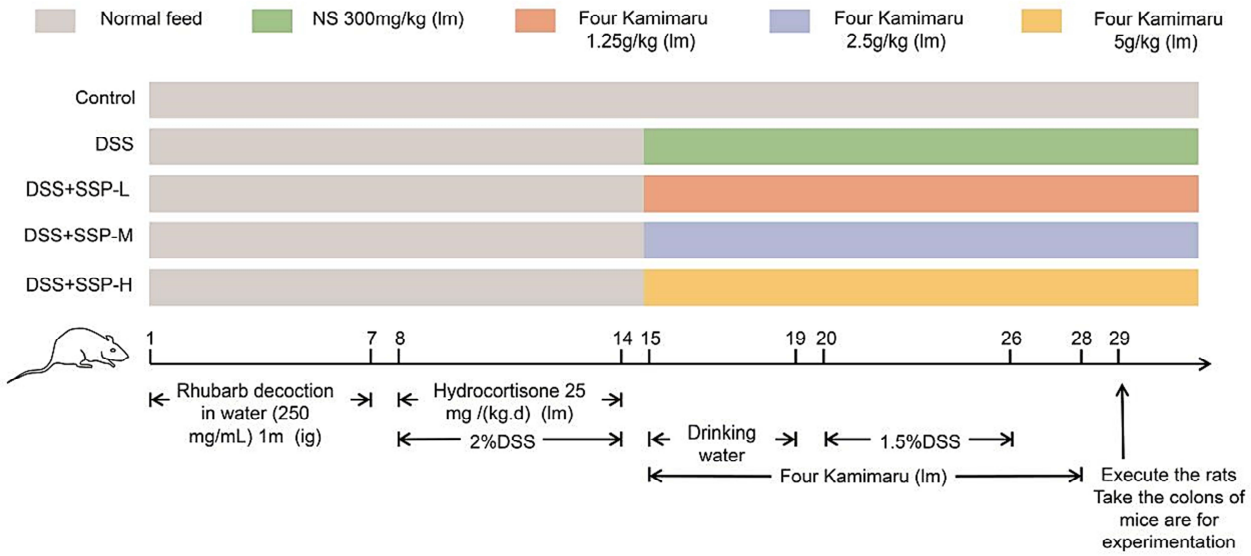


Fig. 1: Schematic diagram for establishing a ulcerative colitis mouse model with spleen-kidney yang deficiency and grouping intervention.

(CCR7+CD45RA-) ratio (P<0.05) and evidently decreased TEM (CCR7-CD45RA-), CCR7-CD45RA-Foxp3+T and CCR7+CD45RA-Foxp3+ ratios in mouse colon tissues (P<0.05), and these processes were dose-dependent (fig. 3). The abovementioned findings suggested that the colon of colitis mice demonstrated a decline in TCM (CCR7+CD45RA-) in colon of colitis mice decreased and an increase in TEM (CCR7-CD45RA-Foxp3+T), TEM (CCR7+CD45RA-Foxp3+) and TEM (CCR7-CD45RA-). SSP might treat colitis by modulating the balance of immunological memory T cell subsets.

Effect of Sishen Pill on inflammatory factor levels in mouse colon tissues with ulcerative colitis of spleen-kidney yang deficiency type

Subsequently, the SSP's effect on inflammatory factor levels in the colitis mouse colon tissues was detected. Briefly, in comparison with the control group, a notable up-regulation was seen in the pro-inflammatory factor (IL-17 and TNF-α) levels in the DSS group (P<0.01). However, in contrast to the DSS group, the DSS+SSP-L, DSS+SSP-M, and DSS+SSP-H groups presented evidently down-regulated IL-17 and TNF-α levels in the mouse colon tissues (P<0.01) and these processes were dose-dependent; in another word, higher DSS doses indicated lower IL-17 and TNF-α levels (fig. 4). Collectively, SSP could considerably alleviate the inflammatory response of colitis mice.

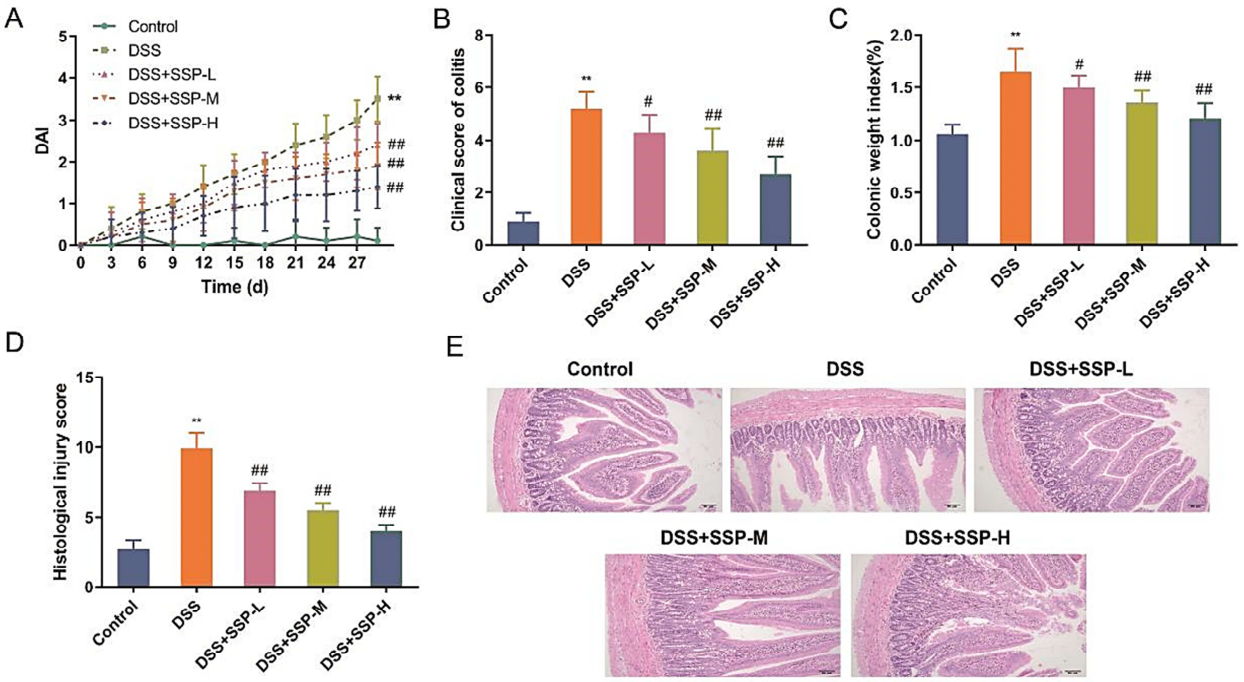
Effect of Sishen Pill on energy metabolism of colitis mouse colon tissues

Finally, the SSP's impact on energy metabolism of colitis mouse colon tissues was assessed. As displayed in fig. 5, the DSS group exhibited a marked decline in ATP and ADP levels relative to the control group, along with the ATP/ADP and ATP/AMP ratios (P<0.01) and a notable elevation in AMP levels (P<0.01). Moreover, in a dose-dependent manner, SSP at varying doses led to a remarkable increase in the ATP and ADP levels, as well as ATP/ADP and ATP/AMP ratios and reduced the AMP level in the DSS-triggered colitis mouse colon tissues (P<0.01, fig. 5). Consequently, DSS could induce energy metabolism disorder in colitis mouse colon tissues, while SSP could effectively alleviate energy metabolism disorder induced by DSS.

DISCUSSION

In this study, rhubarb decoction combined with hydrocortisone was initially used to induce a spleen-kidney yang deficiency model, while DSS was utilized for developing the mouse model of IBDs. In the DSS group mice, severe inflammatory cell infiltration appeared, and extensive ulcers formed and extended to the intestinal wall. After the treatment of SSP, the symptoms of mice were relieved, and the clinical score of colitis, histological injury score and colonic weight index were significantly improved. Hence, SSP is able to effectively treat IBD mice induced by DSS.

Memory T cells are T cells with immunological memory function, which are antigen-specific T cells differentiated from T cells after initial immune response. When the body is stimulated by the same antigen again, memory T cells will function through rapid proliferation and differentiation, produce a strong tolerance to apoptosis, and eliminate infections quickly (Sanchez *et al.,* 2012). According to CCR7 and lymphocyte homing molecules, memory T cells are capable of being allocated into TEM and TCM, with their phenotypes being CD45RA-CD62L+CCR7+ and CD45RA-CD62L-CCR7- (Kumar *et al.,* 2018).



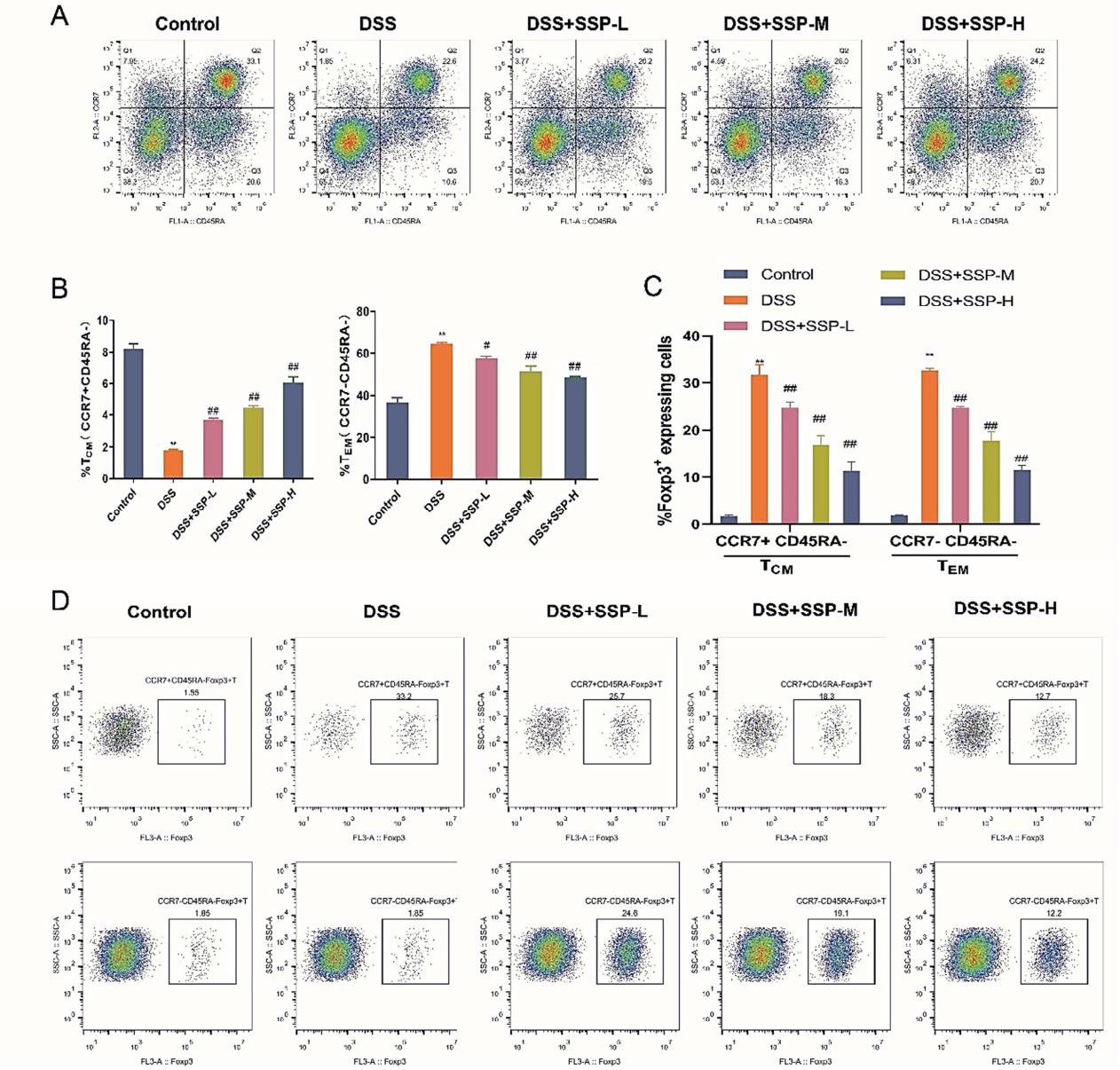
A–D, The DAI score (A), clinical score of colitis (B), colonic weight index (C), and histological injury score (D) in the control, DSS, DSS+SSP-L, DSS+SSP-M, and DSS+SSP-H groups; E, HE staining to evaluate colonic histological injury of mice in each group, the scale bar = 50μm. ##P<0.01 and #P<0.05 *vs*. DSS group; **P < 0.01 *vs*. control group. DSS, dextran sulfate sodium; DAI, disease activity index; SSP, Sishen Pill; H, high; M, medium; L, low.

Fig. 2: Effect of Sishen Pill on ulcerative colitis mouse with a spleen-kidney yang deficiency pattern.

Under normal physiological conditions, chemokines with high expression of TCM can rapidly proliferate after homing to lymphoid tissues and organs; TEM has the function of cell killing, and it can quickly penetrate into inflammatory tissue after secondary immune infection and secrete cytokines to remove infected cells. However, when the cell immune function disorder occurs, TCM will proliferate and differentiate into TEM and secrete more inflammatory factors, thereby inducing toxic reactions and abnormal damage (Espinosa *et al.,* 2016). In addition, TEM (CCR7+CD45RA-Foxp3+) and TEM (CCR7-CD45RA-Foxp3+), as two markers memory T cell subsets, have immunological memory function, higher proliferation and inhibition efficiency, and stronger tissue infiltration ability (Matsuki *et al.,* 2014).

In this paper, the DSS group presented notably declined TCM ratios relative to the control group, along with markedly raised in TEM (CCR7-CD45RA-Foxp3+T), TEM (CCR7+CD45RA-Foxp3+), and TEM (CCR7-CD45RA-) ratios; following treatment with SSP, the above outcomes were reversed. Such outcomes were consistent with the conclusion revealed by a previous study that the role of memory T cell subset imbalance in DSS-induced IBD is related to TCM and TEM. The molecular pathways by which SSP acts on inflammatory responses and immune regulation in IBD appear to involve a dual mechanism: Modulation of inflammatory cytokines and enhancement of cellular energy metabolism SSP's anti-inflammatory effects are largely mediated by down-regulating the TNF-α and IL-17 levels, which contributes to suppressing chronic immune activation. This decrease in pro-inflammatory markers alleviates the inflammatory response, thereby restoring immune equilibrium (Soukou *et al.,* 2018; Liu *et al.,* 2020; Smith *et al.,* 2020). Concurrently, SSP enhances ATP and ADP levels in colon tissues, countering the metabolic deficits characteristic of IBD (Smith *et al.,* 2021). By restoring cellular energy stores, SSP supports epithelial cell function and reduces oxidative stress, ultimately contributing to tissue repair and immune homeostasis. Notably, IBD treatment with SSP is associated with modulation of immunological memory T cell subsets. Also, there was a notable down-regulation in the inflammatory factor (TNF-α and IL-17) levels in mouse colon tissues upon treatment with SSP, as evidenced by the ELISA outcomes.

Energy metabolism is a critical factor in IBD, and the disease is particularly linked to energy metabolism disorders arising from chronic inflammation and intestinal mucosal injury. ATP, ADP, and AMP are important energy molecules in cells. Under normal circumstances, nutrients were oxidized into ATP through the cytochrome oxidase system to serve as the energy needed by cells. Nevertheless, inflammatory response and tissue damage induced by IBD lead to energy metabolism disorder (Smith *et al.,* 2021).



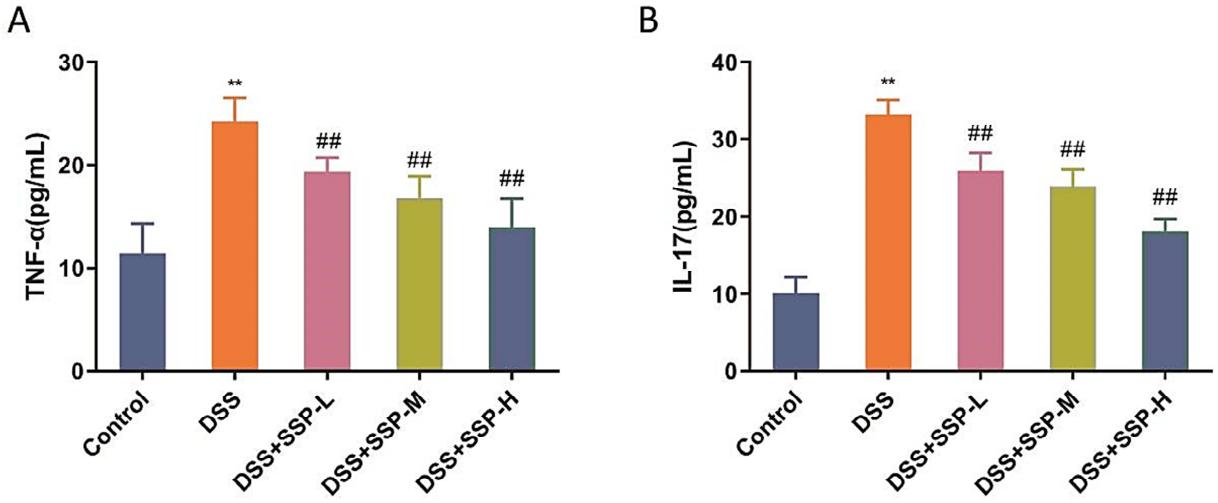
A–D, Flow cytometry for measuring TEM (CCR7-CD45RA-), TCM (CCR7+CD45RA-) (A/B), and Foxp3+ cells (C–D) in mouse colon tissues in the DSS group, DSS+SSP-L group, DSS+SSP-M group, DSS+SSP-H group, and control group. #P<0.05 and ##P <0.01 *vs*. DSS group; **P<0.01 *vs*. control group. TEM, Treg effector memory cell; DSS, dextran sulfate sodium; TCM, Treg central memory cell; SSP, Sishen Pill; H, high; M, medium; L, low; CCR7, CC-chemokine receptor 7.

Fig. 3: Impact of Sishen Pill on immunological memory Treg cell subsets and Foxp3+ cells in ulcerative colitis mice with a spleen-kidney yang deficiency pattern.

In IBD patients, the intestinal mucosa is in an energy-deficient state, marked by low ATP level and energy charge potential (Novak and Mollen, 2015). In this research, the ATP and ADP levels were notably raised in the mouse colon tissues with DSS-triggered IBD, while the AMP level was decreased, which resulted in energy metabolism disorder in the mouse colon tissues. Following treatment with SSP, the ATP and ADP levels were down-regulated. Our outcomes suggested the treatment of IBD by SSP via regulating energy metabolism, consistent with findings from previous research (Garcia and Wilson, 2021).

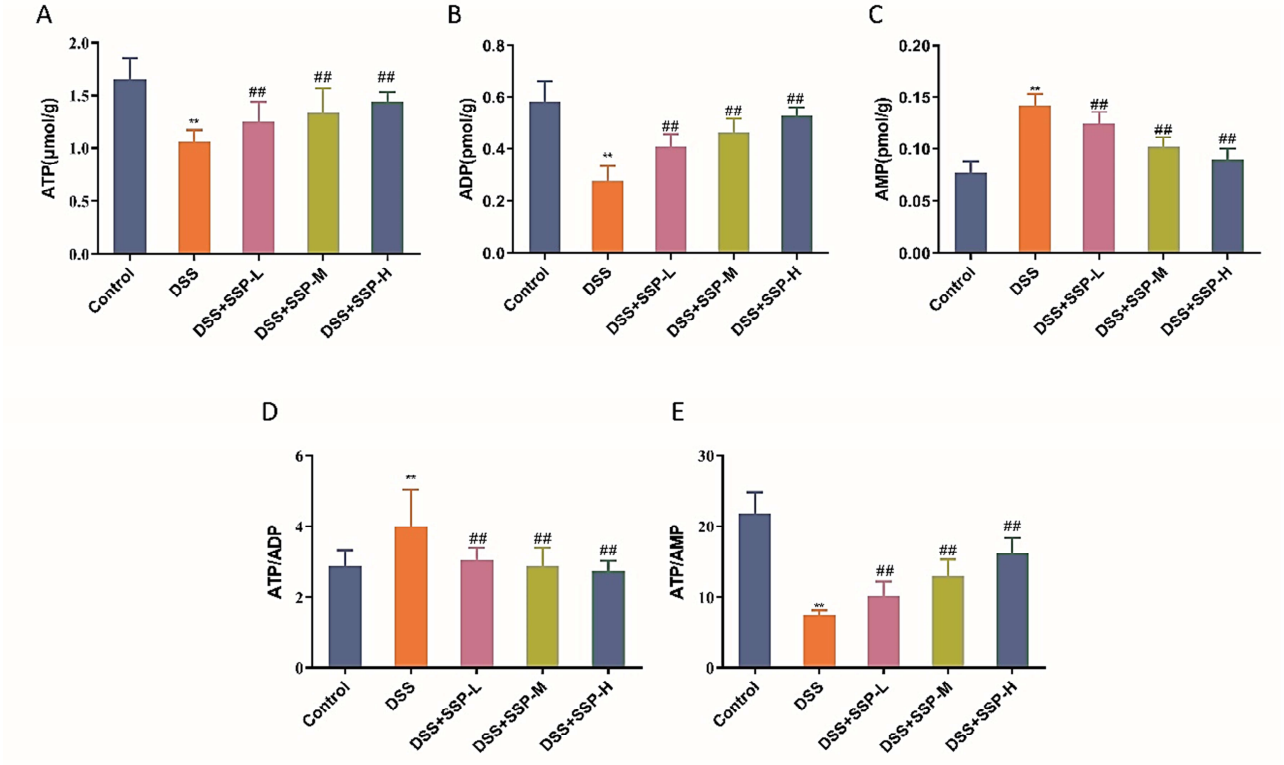
CONCLUSION

SSP may alleviate IBD by balancing memory T cells, inhibiting inflammatory responses, and enhancing energy metabolism. Its effects align with SSP's roles in nourishing "source of fire" and reducing "yin". Specifically, SSP decreases the Foxp3+ T cells and TEM cells (CCR7-CD45RA-) ratios, while increasing TCM cells (CCR7+CD45RA-) in the colon. Additionally, SSP notably enhances energy metabolism by raising the ATP and ADP levels, lowering the AMP level, and enhancing ATP/ADP and ATP/AMP ratios in colon tissues. Yet, the



A–B, ELISA for evaluating the IL-17 and TNF-α levels in mouse colon tissues in the DSS+SSP-H group, DSS+SSP-M group, DSS+SSP-L group, DSS group, and control group. ##P<0.01 *vs*. DSS group; **P<0.01 *vs*. control group. IL, interleukin; DSS, dextran sulfate sodium; SSP, Sishen Pill; TNF-α, tumor necrosis factor alpha; H, high; M, medium; L, low.

Fig. 4: Impact of Sishen Pill on inflammatory factors in colitis mouse colon tissues.



A–C, The ATP (A), ADP (B) and AMP (C) levels, as well as the ATP/ADP (D) and ATP/AMP (E) ratio in the mouse colon tissues in the DSS+SSP-H group, DSS+SSP-M group, DSS+SSP-L group, DSS group, and control group. ##P<0.01 *vs*. DSS group; **P<0.01 *vs*. control group. AMP, adenosine monophosphate; DSS, dextran sulfate sodium; ADP, adenosine diphosphate; SSP, Sishen Pill; ATP, adenosine triphosphate; H, high; M, medium; L, low.

Fig. 5: Effect of Sishen Pill on energy metabolism of colitis mouse colon tissues

specific components and signaling pathways involved remain unclear, necessitating further research.

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