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Research Article

Effect of Osthole on Inflammatory Responses of Mice with Primary Sjögren's Syndrome

Danna ZHANG[1] Jusen YAO[2] Lanying JIANG[1] Bailing YU[1] Chenjie ZHOU[1] Dongyang ZHANG[1] Juan LI [1 (*)]

[1] Department of Geriatrics, Zhejiang Hospital of Integrated Traditional Chinese and Western Medicine, Hangzhou 310003, Zhejiang Province, CHINA

[2] Orthopedics and Traumatology Department of Traditional Chinese Medicine, Hangzhou Tianshui Wulin Street Community Health Care Centre, Hangzhou 310005, Zhejiang Province, CHINA

(*) **Corresponding authors:**
Juan LI

Phone: +86 13067795096
E-mail: lijuan20100810@163.com

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Abstract

We aimed to assess the effect of osthole on the inflammatory responses of mice suffering from primary Sjögren's syndrome (PSS). Fifty naive non-obese diabetic (NOD/Ltj) mice were included to establish a spontaneous model of PSS and assigned into a control group, a 20 mg/kg osthole group, a 30 mg/kg osthole group, a 40 mg/kg osthole group and a 50 mg/kg osthole group in a random manner. Osthole (20, 30, 40, and 50 mg/kg, once a day) together with normal saline (every day) in an equal volume were injected into osthole treatment groups and control group, respectively. Compared with the control group, the inflammatory cells in salivary gland tissues of NOD/Ltj mice manifested gradually ameliorated infiltration, the destruction of acinar cells was relieved gradually ($P<0.05$). In comparison to those obtained from the control group, the inflammatory factors IFN-α, IFN-β, IL-6, IL-8 and TNF-α as well as the GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) in salivary gland tissues of NOD/Ltj mice declined gradually at the expression level as the osthole concentration augmented, showing statistically significant differences ($P<0.05$). Osthole is capable of mitigating inflammatory responses of PSS mice by impeding the cGAS-STING signaling pathway.

Keywords: Osthole, rimary Sjögren's syndrome, inflammatory responses

Introduction

As an autoimmune disease, primary Sjögren's syndrome (PSS) is mainly characterized by symptoms of dryness affecting many organs of the whole body, such as dry mouth, dry eyes, and dry sensation in other parts (such as skin, nose, throat and vagina) [1]. Besides, PSS may also contribute to a series of other symptoms such as joint pain, muscle pain, fatigue, and digestive problems [2]. PSS is mainly ascribed to the decrease in the volume or quality of secretion of glands due to the attack from the immune system, with salivary glands and lacrimal glands as the most common tissues involved [3]. Symptoms of dryness are the major manifestations of PSS, but PSS may also serve as a contributing factor to autoimmune diseases of other systems, such as rheumatoid arthritis and systemic lupus erythematosus [4]. In the clinical treatment of PSS, specific drugs are unavailable currently, and the focus is to relieve symptoms to keep patients comfortable and prevent complications [5]. The regulation of inflammatory response may become one of the important therapeutic strategies for PSS. Inflammation may involve joint tissues, leading to arthritis, and it may also involve muscle tissues, resulting in symptoms such as myalgia and myasthenia [6]. Hence, discovering and developing effective therapeutic drugs in this regard is crucial for PSS patients.

Osthole, a coumarin derivative, exists in several common medicinal plants, such as common Cnidium fruit and Angelica sinensis [7], which can be acquired by extraction and separation from plants or total synthesis. Osthole contains various biological activities such as antineoplastic, cardiovascular protective, neuroprotective, antibacterial, antiparasitic and anti-inflammatory activities [7-10]. Particularly, osthole has been reported to bind multiple

proteins that regulate inflammatory response [11]. Nonetheless, how osthole affects PSS through corresponding molecular regulatory mechanism remains unclear.

Hence, we herein assessed the effect of osthole on inflammatory responses on PSS mice, aiming to provide an experimental foundation for treating PSS.

Materials And Methods

Ethical Approval

This study was conducted after approval by our hospital's animal ethics committee (Approval No. ZHITCWM2022045), and great efforts have been made to minimize the animals' suffering.

Experimental Materials and Apparatus

Male naive non-obese diabetic (NOD/Ltj) mice (6 weeks old, n=50) were bought from the Model Animal Research Center of Nanjing University. Osthole (specification: 1 g/vial, purity: HPLC ≥98%, batch No.: 220508) was acquired from Sichuan Vicky Biotechnology Co., Ltd. (China). The normal saline, bicinchoninic acid (BCA) kits, formaldehyde, skimmed milk powder (5%), paraffin, ethanol, neutral gum, radioimmunoprecipitation assay (RIPA) lysis buffer, Tris-buffered saline-Tween 20 (TBST) and enhanced chemiluminescence (ECL) kits were bought from Solarbio (Beijing, China). A hematoxylin-eosin (HE) staining kit sourced from Beyotime (Shanghai, China). The TRIzol reagent, a PrimeScript reverse transcription (RT) Master Mix kit was offered by TaKaRa (Japan) together with a SYBR® Premix Ex Taq™ quantitative assay kit. A polyvinylidene fluoride (PVDF) membrane was provided by Invitrogen (USA). All antibodies were offered by Abcam. An optical microscope was purchased from Leica (Germany).

Feeding and Grouping of Animals

All male NOD/Ltj mice were adaptively fed for one week in cages (5 mice/cage, humidity: 50-70%, temperature: 22-24°C) with a 12/12 h light/dark cycle. Moreover, enough food and water were offered to ensure that mice could take food and water freely. The Guide for Care and Use of Laboratory Animals was set as the standard for all assays on mice [12]. All treatments started when mice were 8 weeks old: Five groups were determined for equal and random allocation of the 50 NOD/Ltj mice: a control group, a 20 mg/kg osthole group, a 30 mg/kg osthole group, a 40 mg/kg osthole group and a 50 mg/kg osthole group. Mice in the osthole treatment groups were injected with osthole (20, 30, 40, and 50 mg/kg) once a day, while normal saline was injected in an equal volume to those in the control group every day. The salivary volume of mice in each group was measured at Weeks 2, 3 and 4 after treatment. Following 4 weeks of treatment, mice were euthanized to collect the salivary gland tissues for further detection.

Observation of Salivary Gland Tissues by HE Staining

Histological examination was conducted on salivary gland tissue samples of the submandibular gland of mice using the HE staining kit. Briefly, salivary gland tissues were successively subjected to fixation in formaldehyde, dehydration, and embedding in paraffin. Thereafter, paraffin-embedded tissues were sliced into tissue sections (4 μm in thickness) and then stained with HE. Following dehydration with gradient ethanol, the sections were fixed with neutral gum. Finally, the optical microscope was employed to observe the staining results.

Observation of Variations of the Liver

At Week 4 following treatment, the liver was taken out from the euthanized mice, weighed and photographed. Furthermore, HE staining was carried out to observe the morphology of stem cells in liver tissues.

Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR) Analysis of Inflammatory Factor Expressions

Salivary gland tissues collected from each group of mice were ground, followed by qRT-PCR analysis on inflammatory factors to obtain their expression levels by reference to the methods adopted by Zeng et al.[13]. According to the steps in the instructions, total RNA extraction from mouse salivary gland tissues was conducted using TRIzol reagent. Subsequently, the PrimeScript RT Master Mix kit was employed to obtain cDNA from the extracted total RNA through reverse transcription. Afterwards, using following the instructions of the SYBR® Premix Ex Taq™ quantitative assay kit, qRT-PCR was carried out to measure the gene expression levels of IFN-α, IFN-β, IL-6, IL-8 and TNF-α in salivary gland tissues. The internal reference determined as GAPDH, the 2^{-Ct} method was adopted for the calculation of gene relative expressions. *Table 1* presents the sequences of primers utilized.

Measurement of Levels of Cyclic GMP-AMP Synthase (cGAS)-Stimulator of interferon Genes (STING) Signaling Pathway-Associated Proteins by Western Blotting

Salivary gland tissues collected from mice in each group were ground and then detected for the content of cGAS-STING signaling pathway-associated proteins through Western blotting by reference to the methods utilized by Lyu et al.[14]. The isolation of total protein was completed through lysis of the homogenate of salivary glands using the RIPA lysis buffer. Then the content of the isolated total protein was quantified by BCA kit. Next, 10% sodium

Table 1. Sequences of primers used in qRT-PCR

Name	Primers for PCR (5'-3')	
IFN-α	Forward	GCCTCGCCCTTTGCTTTACT
	Reverse	CTGTGGGTCTCAGGGAGATCA
IFN-β	Forward	ATGACCAACAAGTGTCTCCTCC
	Reverse	GGAATCCAAGCAAGTTGTAGCTC
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCAGGTTG
IL-8	Forward	TGTTCACAGGTGACTGCTCC
	Reverse	AGCCCATAGTGGAGTGGGAT
TNF-α	Forward	AGGCACTCCCCCAAAAGATG
	Reverse	CCACTTGGTGGTTTGTGAGTG
GAPDH	Forward	ACAGCAACAGGGTGGTGGAC
	Reverse	TTTGAGGGTGCAGCGAACTT

dodecyl sulfate polyacrylamide gel for protein lysate separation was performed, followed by PVDF membrane transfer. Subsequently, the TBST buffer mixed with 5% skimmed milk powder was added for room-temperature blocking of the PVDF membrane for 1 h. Afterwards, the membrane was added with primary antibodies (anti-cGAS, anti-STING and anti-β-actin) for incubation at 4°C overnight. Following membrane washing, 2 h of incubation with secondary antibodies labeled with horseradish peroxidase was completed. Finally, the ECL kit was used to detect protein signals, with the results analyzed by ImageJ software.

Statistical Analysis

GraphPad Prism 8.0 software was utilized for statistical analysis. For each experiment, three replicates were set. Mean ± standard deviation ($\bar{x}\pm s$) was selected as the format to express all data, and the comparison among groups and pairwise comparison between groups were completed through analysis of variance and LSD-*t* test, respectively. The differences with statistical significance were indicated by $P<0.05$.

Results

Effect of Osthole on Disease Progression of Salivary Gland Tissues in NOD/Ltj Mice

Compared with the control group, both the penetration of inflammatory cells and the destruction of acinar cells in salivary gland tissues of NOD/Ltj mice were gradually ameliorated with the increase in osthole concentration, implying that for NOD/Ltj mice, osthole impedes the disease progression of salivary gland tissues *(Fig. 1)*.

Effect of Osthole on Salivary Volume in NOD/Ltj Mice

In comparison to the control group, the saliva volume secreted by salivary gland tissues rose bit by bit in NOD/Ltj mice with the increase of osthole concentration, with a difference of statistical significance ($P<0.05$) *(Table 2)*.

Effect of Osthole on the Liver of NOD/Ltj Mice

No obvious variations were observed in the texture and weight of the liver of NOD/Ltj mice treated with osthole in comparison to those obtained from the control group ($P>0.05$). Besides, the observation results of liver stem cells of mice in the 50 mg/kg osthole group uncovered that these cells displayed normal morphology. These results demonstrated that osthole has no significant effect on NOD/Ltj mice's liver *(Fig. 2)*.

Role of Osthole in Influencing Inflammatory Factors in NOD/Ltj Mice from the Aspect of Expression Level

The inflammatory factors IFN-α, IFN-β, IL-6, IL-8 and TNF-α gradually dropped at the expression level with the augmentation of osthole concentration, with statistically significant differences by contrast to those in the control group ($P<0.05$). This suggested that osthole represses inflammatory factors in salivary gland tissues of NOD/Ltj mice in terms of their expressions *(Fig. 3)*.

Effect of Osthole on cGAS-STING Signaling Pathway-Associated Protein Levels in NOD/Ltj Mice

When the osthole concentration augmented, the NOD/Ltj mice, contrasted with the control group, exhibited reduced expression levels of cGAS and STING in salivary

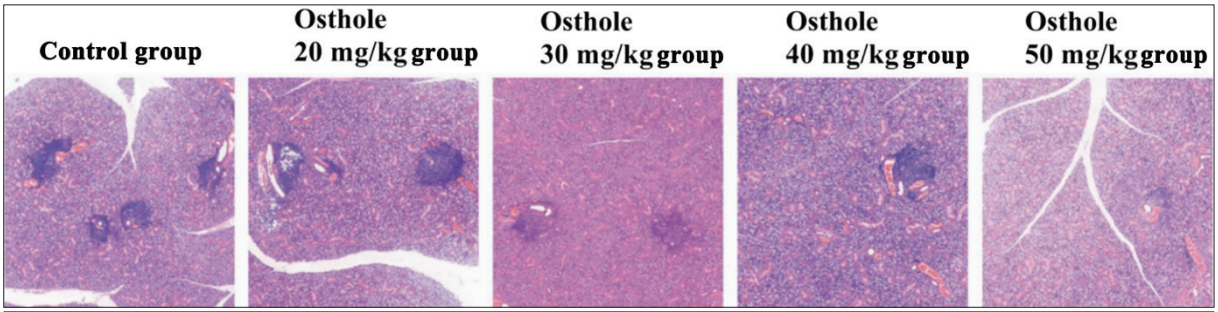

Fig 1. Effect of osthole on disease progression of salivary glands in NOD/Ltj mice. Salivary gland tissues from the submandibular gland in mice observed by HE staining

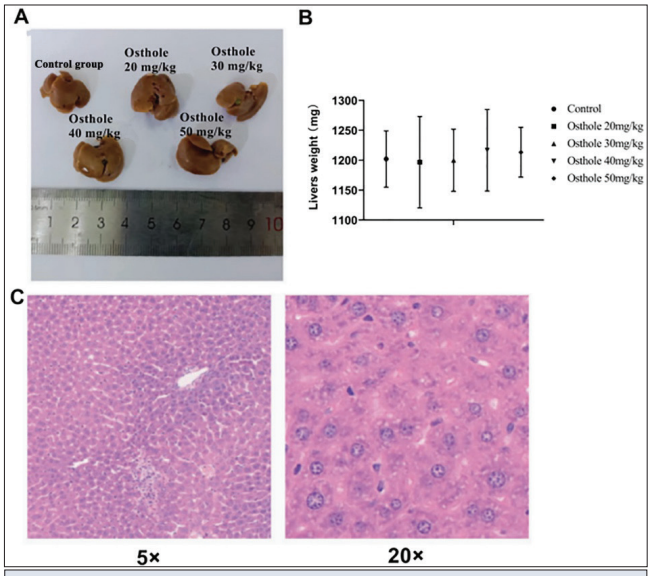

Fig 2. Impact of osthole on NOD/Ltj mice's liver. A-B: Comparison of liver texture and weight of NOD/Ltj mice among groups. C: Histological morphology of liver stem cells in the 50 mg/kg osthole group observed by HE staining

gland tissues in a gradual manner, with differences of statistical significance (P<0.05). This implied that regarding NOD/Ltj mice, osthole inhibits cGAS-STING signaling pathway-associated protein expressions in salivary gland tissues (*Fig. 4*).

Table 2. *Salivary volume of mice ($\bar{x}\pm s$, mg/min)*

Group	n	Week 2	Week 3	Week 4
Control	10	6.21±1.86	6.32±1.52	6.42±1.73
20 mg/kg osthole	10	9.03±1.79^{a}	10.87±1.57^{a}	12.36±1.96^{a}
30 mg/kg osthole	10	11.37±1.64^{ab}	13.56±2.32^{ab}	14.72±1.35^{ab}
40 mg/kg osthole	10	14.13±2.87^{abc}	15.05±2.48^{abc}	17.56±1.78^{abc}
50 mg/kg osthole	10	17.23±2.52^{abcd}	18.86±2.27^{abcd}	19.93±2.07^{abcd}

a *P<0.05 vs. control group,* ***b*** *P<0.05 vs. 20 mg/kg osthole group,* ***c*** *P<0.05 vs. 30 mg/kg osthole group,* ***d*** *P<0.05 vs. 40 mg/kg osthole group*

Discussion

PSS is an autoimmune disease mediated by the immune system, with its pathogenesis involving the abnormal attack on the own tissues from the immune system [15]. In the case of PSS, the attack and destruction from the immune system negatively affect the salivary glands, lacrimal glands and other secretory gland tissues to weaken or eliminate the secretory function of these glands, thus inducing such symptoms of dryness as dry mouth and dry eyes [16]. Inflammatory responses are crucial participants in PSS pathogenesis. After abnormal activation of the immune system, chemokines, cytokines and other inflammatory mediators are delivered, resulting in inflammatory responses. These inflammatory mediators attract and

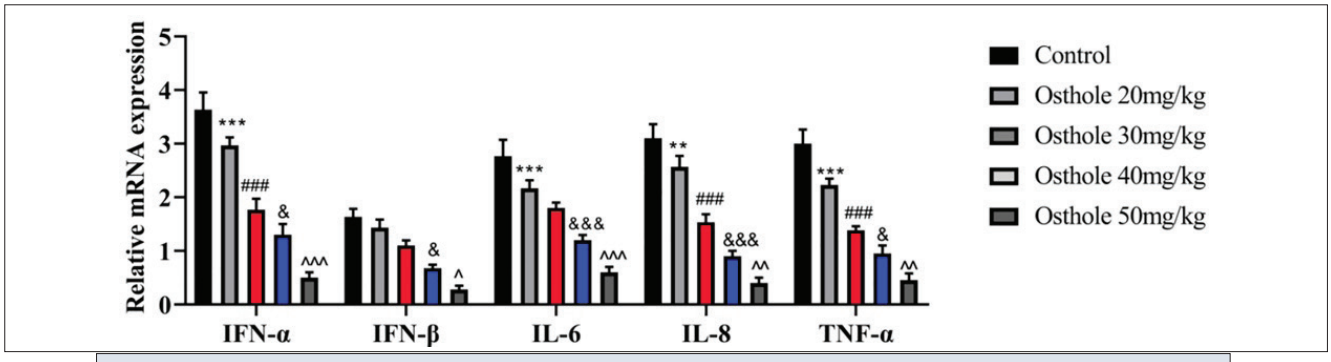

Fig 3. Role of osthole in influencing inflammatory factors in NOD/Ltj mice at the expression level. Expressions of inflammatory factors in murine salivary gland tissues in each group analyzed by qRT-PCR. **P<0.01, ***P<0.001 *vs.* Control Group, $^{###}$P<0.001 *vs.* 20 mg/kg osthole Group, $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 *vs.* 30 mg/kg osthole Group, $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01, $^{\wedge\wedge\wedge}$P<0.001 *vs.* 40 mg/kg osthole Group

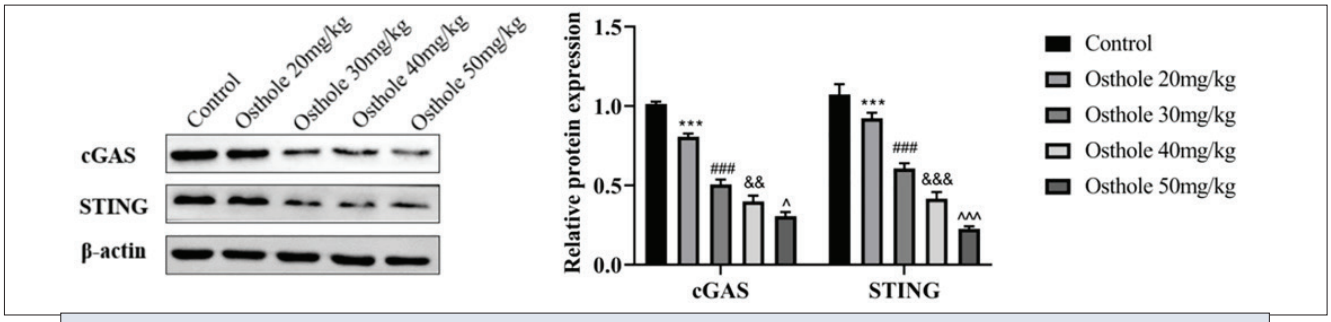

Fig 4. Effect of osthole on cGAS-STING signaling pathway-associated protein content in NOD/Ltj mice. Western blotting for cGAS-STING signaling pathway-associated protein concentrations in salivary gland tissues of mice in each group. ***P<0.001 *vs.* Control Group, $^{###}$P<0.001 *vs.* 20 mg/kg osthole Group, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 *vs.* 30 mg/kg osthole Group, $^{\wedge}$P<0.05, $^{\wedge\wedge\wedge}$P<0.001 *vs.* 40 mg/kg osthole Group

activate inflammatory cells, such as monocytes and lymphocytes, thereby leading to inflammatory responses in local tissues [17]. In the case of PSS, inflammatory responses are mainly observed in the tissues around the affected glands, giving rise to gland damage and hypofunction [18]. In addition to the direct effect on the secretory glands, inflammatory responses may also be responsible for inflammation and injury of other systems and organs, resulting in a series of other symptoms and complications. Accordingly, it is crucial to know the relationship between PSS and inflammatory responses for the purpose of understanding the pathogenesis of PSS, as well as for its diagnosis and treatment.

Liu et al.[19] reported that melatonin repressed the hypofunction and inflammation progression of salivary glands in NOD/Ltj mice by modulating immune response, finally mitigating Sjögren's syndrome-like symptoms. In the present study, 50 NOD/Ltj mice used as spontaneous animal models of PSS were assigned into a control group as well as osthole 20, 30, 40 and 50 mg/kg groups at random. It was uncovered that the destruction of acinar cells relieved in a gradual manner, the infiltration of inflammatory cells in salivary gland tissues of NOD/Ltj mice gradually meliorated, and the volume of saliva secreted by salivary gland tissues rose bit by bit with the increase in osthole concentration, and they were statistically significantly different from those in the control group ($P<0.05$). This suggested the inhibitory effect of osthole on disease progression of salivary gland tissues in NOD/Ltj mice, comparable to the results of Liu et al.[19]. Besides, the osthole treatment groups compared to the control group exhibited no obvious variations in the texture and weight of the liver ($P>0.05$). Furthermore, it was observed that liver stem cells of mice in the 50 mg/kg osthole group displayed normal morphology. These results signified that osthole has no significant effect on the liver of NOD/Ltj mice, with good drug safety.

Osthole has been reported to exert a good anti-inflammatory effect [20]. For instance, a study conducted by Kordulewska et al.[21] manifested that osthole could alleviate gastrointestinal inflammation by impeding pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) from secretion. Moreover, Wang et al.[22] discovered that osthole could modulate the polarization to M1 macrophages induced by lipopolysaccharides, reducing inflammatory responses. In this study, as the osthole concentration augmented, NOD/Ltj mice displayed progressively lowered the expression levels of such inflammatory factors as IFN-α, IFN-β, IL-6, IL-8 and TNF-α in salivary gland tissues compared with the control group, presenting statistically significant differences ($P<0.05$). This verified the ability of osthole to suppress the expression of inflammatory factors in salivary gland tissues of NOD/Ltj mice, echoing research findings by Kordulewska et al.[21] and Wang et al.[22].

As a crucial immune signaling pathway, the cGAS-STING signaling pathway serves as a vital player in resisting pathogenic microorganisms and infections and regulating autoimmune balance [23]. The cGAS-STING signaling pathway under activation can promote immune cells to respond to virus infection and DNA damage, thus regulating inflammatory responses and antiviral immunity [24]. Nevertheless, excessive activation or abnormal regulation of such a pathway may also give rise to such diseases as autoimmune diseases and inflammatory diseases. For this reason, the regulation of balance in this pathway is crucial for the maintenance of immune balance and prevention of diseases [25]. As reported by Zhou et al.[26], the activated cGAS-STING signaling pathway had a close correlation with the pathogenesis of PSS. Rapamycin is found to be able to alleviate submandibular gland lesions in mice with Sjögren's syndrome by repressing the cGAS-STING signaling pathway from activation. It was uncovered through this research that compared to the control group, the elevation of osthole concentration gradually lowered cGAS plus STING expression levels in salivary gland tissues of NOD/Ltj mice, and there were differences with statistical significance ($P<0.05$). This demonstrated that osthole impedes the protein expression associated with the cGAS-STING signaling pathway in salivary gland tissues of NOD/Ltj mice, thereby exerting its anti-inflammatory effect. To sum up, it was discovered in this study for the first time that osthole can mitigate the inflammatory responses of PSS mice by suppressing the cGAS-STING signaling pathway, which renders a novel idea for curing PSS. However, some shortcomings also exist in this research. For example, the effect of osthole was only studied using NOD/Ltj mouse models. Therefore, subsequent clinical studies are required to validate conclusion of this work.

In conclusion, osthole can relieve inflammatory responses in PSS mice by inhibiting the cGAS-STING signaling pathway, suggesting that osthole probably acts as a potential drug effective for PSS.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Ethical Approval: This study has been approved by the animal ethics committee of our hospital (approval No. ZHITCWM2022045), and great efforts have been made to minimize the animals' suffering.

Competing Interests: There is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The article, tables and figures were not written/created by AI and AI-assisted technologies.

Authors' Contributions: D. Zhang and J. Yao designed and performed the study, and drafted the paper; J. Li conceived and supervised the study, and significantly revised the paper; L. Jiang, B. Yu, C. Zhou and D. Zhang performed and analyzed the experiments. All authors have approved the submission and publication of this paper.

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