Effects of Multi-glycosides of *Tripterygium wilfordiion* in the Treatment of Sjögren's Syndrome in the Non-obese Diabetic Mouse Model

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Objective: *To investigate the effects of the multi-glycosides of Tripterygium wilfordii (GTW) on Sjögren's syndrome (SS) in the non-obese diabetic (NOD) mouse model.*
Methods: *Twenty-seven 8-week-old, female NOD mice were divided into the GTW group, the hydroxychloroquine (HCQ) group, and control (normal saline) group, and received corresponding treatment for 16 weeks. The treatment-induced changes in stimulated total saliva flow rate (STFR), level of serum anti-SSA/SSB, ratio of regulatory T (Treg) cells, histology of the submandibular gland (SMG) and the gene expression profile that is related to inflammation and autoimmunization were evaluated.*
Results: *Compared to the untreated (control) mice, STRF, SMG index and Treg/CD4+ cell ratio were significantly higher, whereas anti-SSA, anti-SSB and lymphoid foci were remarkably lower in GTW-treated mice. HCQ-treated mice showed similar results except SMG index was not different from the untreated mice. NOD mice showed 19.03% altered gene expression with maturation from the age of 8 weeks to 24 weeks. Treatment with HCQ and GTW reduced the change in gene expression to 13.09% and 7.14%, respectively.*
Conclusion: *GTW is as effective as HCQ in the treatment of Sjögren's syndrome in the NOD mouse model.*

Key words: *multi-glycoside of Tripterygium wilfordii, NOD mouse model, Sjögren's syndrome*

Sjögren's syndrome (SS) is one of the most common chronic autoimmune diseases with a prevalence of 0.4% to 0.7%[1,2]. The characteristic clinical manifestation of SS is the ocular and oral dryness due to the progressive destruction of lacrimal and salivary glands. Although seldom fatal, SS dramatically diminishes the patients' quality of life. The exocrinopathy of SS may occur alone (primary SS) or in association with other autoimmune disorders (secondary SS)[3-5]. Since the aetiology of SS remains undefined, effective therapies remain lacking. The current treatment or management is mainly palliative, using symptomatic interventions. The most frequently used therapy is stimulation of muscarinic acetylcholine receptors with pilocarpine or cevimeline, which has shown some level of effectiveness. The other therapies include immunosuppression with corticosteroids and B cell depletion with rituximab. All these therapies have considerable side effects, which hinder the acceptance of the treatments by patients with SS. Another commonly used immunosuppressive drug is hydroxychloroquine (HCQ)[6]. Similarly, as shown in other drugs for the treatment of SS, the efficacy of HCQ in the treatment of SS appears to be unsatisfactory. In a recent randomised, double-blind and placebo-controlled clinical trial involving 120 SS patients over 24

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weeks, treatment with HCQ did not indicate substantial improvement in the symptoms of SS, including dryness, pain and fatigue[7]. In addition, severe side effects such as retinal damage and pigmentary retinopathy significantly limited its long-term application[8].

The multi-glycoside of *Tripterygium wilfordii* (GTW) is a substance extracted from the peeled roots of a traditional Chinese herb medicine, Tripterygium wilfordi, which has been widely used in the treatment of various autoimmune and inflammatory diseases. Treatment of several autoimmune disorders such as rheumatoid arthritis and lupus with GTW has a long history in traditional Chinese medicine. It has been demonstrated that GTW inhibits the expression of pro-inflammatory cytokines, adhesion molecules and matrix metalloproteinases by macrophages, lymphocytes, synovial fibroblasts and chondrocytes. GTW also inhibits the proliferation of lymphocytes by inducing apoptosis, reduces serum IgG level and corrects the imbalance of T-lymphocyte subsets[9-11]. A number of prospective, double-blind, randomised and placebo-controlled clinical trials have shown that treatment with GTW significantly improved rheumatoid arthritis disease activity.

Since SS has many common characteristics in pathogenesis with lupus and rheumatoid arthritis, we hypothesise that GTW is likely to have the potential to reduce autoimmunity, improve SS symptoms and protect salivary gland tissue and function. Therefore, the present study aimed to test this hypothesis by using the non-obese diabetic (NOD) mouse model of Sjögren's syndrome. NOD mice spontaneously develop lacrimal and salivary gland autoimmunity and are a well-characterised animal model for Sjögren's syndrome[12]. It has been well established that the onset of SS-like diseases in NOD mice is usually at the age of 6 to 10 weeks, and the disease is fully developed after the age of 20 weeks[3-5].

Materials and methods

Animal care and treatments

Eight-week-old female NOD mice were purchased from Shanghai Slac Laboratory Animal Co and held in the Health Science Center Animal Care Facility of Peking University. The experiments and animal treatment procedures were approved by the Institutional Ethics Committee of Peking University.

Twenty-seven NOD mice were randomly divided into three groups; the GTW, HCQ and control group, with nine mice in each. Mice in the GTW group were intragastrically given 5 mg/kg/day GTW (Hunan Xieli Pharmaceutical Co, China). Mice in the HCQ group were given 50 mg/kg/day HCQ (Shanghai Zhongxi Pharmaceutical Co, China). The control group received the same volume of normal saline (NS) every day. The experimental treatment lasted for 16 weeks. The dosage of GTW and HCQ was selected so that it was equivalent to the dose used in humans[13].

Measurement of salivary flow rate

Saliva flow rate was measured every other week. In brief, the mice were anesthetised by intraperitoneal injection of 0.36 g/kg body weight of tribromoethanol (Alfa Aesar, Massachusetts, USA). Five minutes later, 0.5 mg/kg body weight of pilocarpine (Sigma, Missouri, USA) was intraperitoneally injected to stimulate saliva secretion. Whole saliva was collected for 15 min and the stimulated total saliva flow rate was calculated according to a standard protocol used in our previous study[14].

Autoantibody assessment

Blood of the NOD mice were harvested from the inner canthus at the age of 10, 16 and 24 weeks. IgG class autoantibodies against the ribonuclear proteins SSA/Ro and SSB/La (Euroimmun, Lübeck, Germany) in the serum were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol.

Histological examination of the submandibular gland

The submandibular glands (SMGs) were quickly discerned within 10 min after the mice were killed. The weight of the glands was measured and SMG index was calculated by comparing the weight of the glands and the bodyweight of the mice (SMG index = SMG weight/body weight)[15]. The specimens were then fixed in 10% formalin, dehydrated in graded ethanol and embedded in paraffin. Histological sections (5 μm thick) were prepared, stained with H&E staining and examined with a microscope for lymphocyte infiltration of the tissues. Clusters of ≥ 50 lymphocytes in a 4 mm^2 area were considered as abnormal[16].

Flow cytometric analysis

The spleen monocytes were immediately harvested after the mice were killed. The cells were stained with anti-CD4-FITC (BD PharMingen, California,

USA), and incubated, permeabilised in Fix/Perm buffer (eBioscience, California, USA), and stained with anti-FoxP3-PE (eBioscience, California, USA). The isotype-matched control antibody was used in FACS analyses. Cells were analysed on an FACS Calibur flow cytometer using Cell Quest software (Becton Dickinson, California, USA).

Microarray analysis

Three mice in each group were randomly selected and spleen T cells were harvested for microarray analysis. In addition, spleen T cells of three 8-week-old mice, whom had not received any treatment were obtained as the baseline. Eighty-four cytokine genes, including the interferon (IFN), interleukin (IL), TGF-β families and the TNF superfamily related to inflammation and autoimmunization, were chosen for analysis.

Total RNA was extracted from 1×10^6 spleen T cells with TRIZOL (Invitrogen life technologies, California, USA). DNase was used to remove the contamination during preparation. An optical density (OD) assay was performed to calculate the concentration and 1% agarose gel to check the quality of RNA product. First strand cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR) were conducted using super-array PCR master mix (SA Biosciences, QIAGEN, Hilden, Germany). 2^(ΔΔCt) method was used to analyse the data obtained from each array[17].

Examination of side effects

All animals were monitored carefully to detect any clinically abnormal behaviours or activities. The body weight of all mice were evaluated at the age of 8, 16 and 24 weeks. Histological changes in the liver and kidney were evaluated by histological examinations.

Statistical analysis

The data was expressed as mean ± standard deviation and analysed with ANOVA using SPSS 11.5 software (SPSS Base 11.5, Wacker Drive, Illinois, USA). The statistical significance level was set as $P < 0.05$.

Results

Side effect

The HCQ- and GWT-treated mice were closely monitored for any adverse physical reactions or behavioural activities during the experimental treatments. HCQ-treated mice showed depilation and swelling in five out of nine mice after 16 weeks of age, while no similar symptoms were found in the GTW-treated mice and control mice during the experimental period. No drug-induced liver and kidney toxicity were observed in all three animal groups.

Table 1 Body weights of mice in the different groups (g).

Group	8 week	16 week	24 week
Control	19.72 ± 0.95	22.48 ± 1.01	22.94 ± 0.87
HCQ	19.72 ± 0.95	22.46 ± 1.82	23.43 ± 2.70
GTW	19.72 ± 0.95	22.02 ± 1.40	22.66 ± 2.10

Effects of GTW and HCQ on NOD mouse body weights

Body weights of the mice in three groups were slightly increased during the experimental treatment (Table 1). However, the increase was small; only 3 to 4 g during the 16 weeks in all three groups. This phenomenon is likely due to the influence of spontaneously occurring SS and diabetes[3-5]. There was no significant difference in body weights amongst three groups (Table 1), suggesting that body weight is not affected by TGW or HCQ treatment.

Effects of GTW and HCQ on saliva flow rate

The pilocarpine-stimulated whole saliva flow rate was significantly reduced in the control mice beginning from the tenth week of age (Fig 1). This presents a piece of clear and solid evidence demonstrating that salivary glands are involved and the secretion function of salivary glands was significantly reduced. However, the rates of salivary secretion in the mice of both the GTW group and the HCQ group were dramatically higher compared to the rate in the untreated mice, beginning from the tenth week of age (Fig 1). There were no substantial differences in the whole saliva flow rates between the GTW group and the HCQ group.

Effects of GTW and HCQ on the expression of autoantibodies

The increased autoantibody expression is a characteristic of SS. The expression of anti-SSA/Ro and anti-SSB/La was gradually elevated in the untreated NOD (control) mice, indicating that the severity of the autoimmune disorder is increased with time (Fig 2). Compared to the

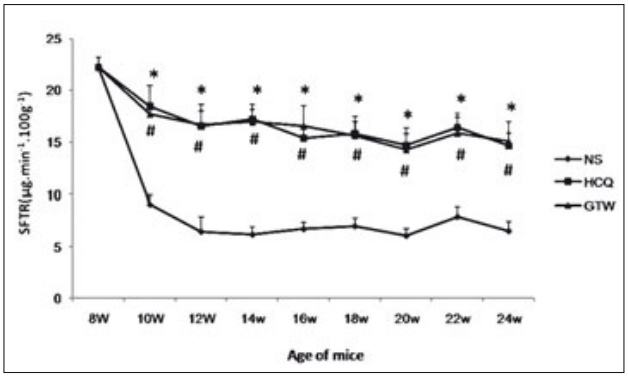

Fig 1 Stimulated whole salivary flow rates of the NOD mice in the control and treatment groups at different time points. Stimulated whole saliva flow rates in HCQ- and GTW-treated mice were significantly higher than in the control group. *$P < 0.05$ between the GTW group and the control group; #$P < 0.05$ between the HCQ group and the control group.

control group, the autoantibody concentrations of anti-SSA/Ro and anti-SSB/La were significantly lower at 16 and 24 weeks in both the GTW and the HCQ groups (Fig 2). There was no significant difference between the GTW and HCQ group.

Effects of GTW and HCQ on SMG index

At the end of the experimental treatment (24 weeks of age), the SMG index of the control group was 6.30 ± 0.96 (n = 9). The SMG index in the GTW group was 7.55 ± 1.08 (n = 9), significantly higher than that in the control group ($P < 0.05$). However, there was no substantial difference in SMG indexes between the HCQ group (6.78 ± 0.77; n = 9) and the control group ($P > 0.05$).

Effects of GTW and HCQ on SMG histology

The SMG histology was examined in the GTW- and HCQ-treated mice and compared with the untreated control mice. The characteristics of inflammation were observed in all mice at the age of 24 weeks. The major change was the lymphocyte infiltration in the SMG tissues. The lymphocytes were located mainly around the blood vessels and salivary ducts. The structure of acini were completely destroyed in severe infiltration areas (Fig 3A). The lymphoid foci in control mice were 7.33 ± 1.22 (n = 9; Fig 3A). Treatment with GTW significantly reduced lymphocyte foci (2.67 ± 1.22; n = 9; $P < 0.05$; Fig 3B). Similarly, treatment with HCQ also significantly reduced lymphocyte infiltration (2.22 ± 0.83; n = 9; $P < 0.05$; Fig 3C). There was no significant difference between GTW and HCQ groups.

Effects of GTW and HCQ on Treg cells in the spleen

The Treg ($CD4^+CD25^+FoxP3^+$) cells are required to maintain immunological tolerance and population change or dysfunction of Treg cells is a major characteristic of autoimmunity. After 24 weeks of treatment of the NOD mice, the Treg cell ratio was 15.11 ± 3.71% (n = 9). Treg T cell ratio in GTW-treated NOD mice was significantly higher (21.39 ± 3.01%; n = 9; $P < 0.05$). Similarly, the ratio in HCQ-treated NOD mice was also significant-

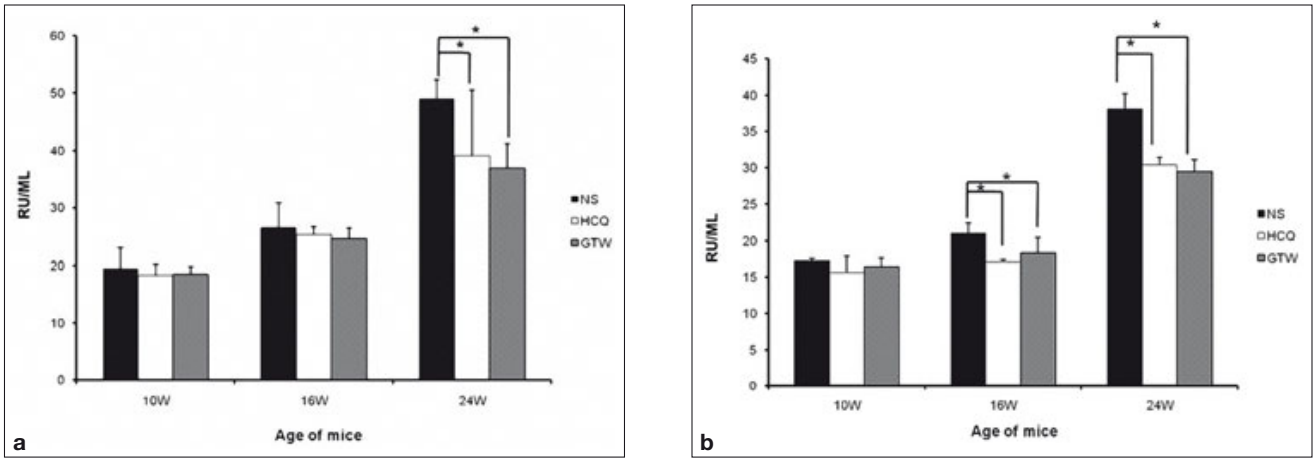

Fig 2 Serum levels of autoantibodies of NOD mice. The titers of serum anti-SSA antibody (a) and anti-SSB antibody (b) were increased in all groups along with the age, at 16 and 24 weeks. The titers of SSB in HCQ and GTW groups were significantly lower than the control group. No significant difference between the GTW and HCQ group was found. RU: relative unit. *$P < 0.05$.

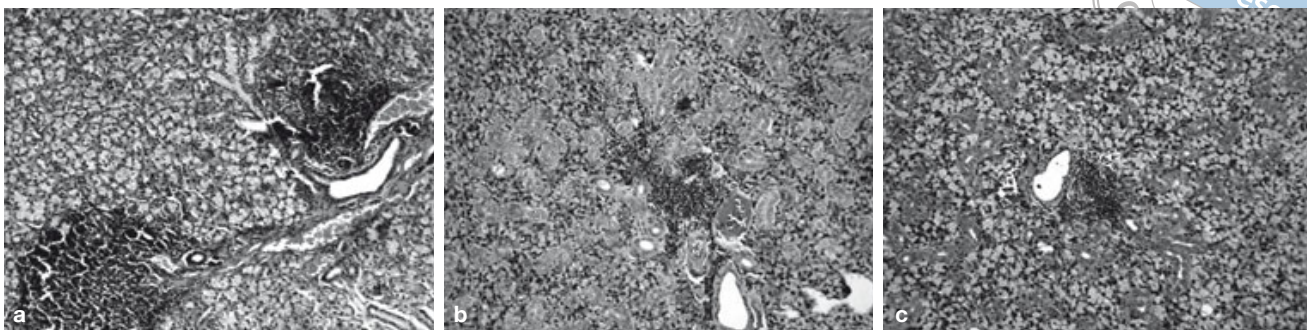

Fig 3 Histological examination of submandibular glands of NOD mice in (a) control, (b) HCQ and (c) GTW groups (H and E staining, original magnification ×20). Lymphocytic infiltration was significantly reduced in mice treated with HCQ and GTW.

ly higher (20.88 ± 2.27%; n = 9; $P < 0.05$). However, no significant difference was observed between the GTW group and the HCQ group ($P > 0.05$).

Effects of GTW and HCQ on gene expression

A comparison between the gene expression profiles in untreated (control) NOD mice at the age of 8 weeks and 24 weeks showed that, amongst a total of 84 genes tested, 16 genes (13 upregulated and 3 downregulated) showed altered expression after maturation, accounting for 19.03% of the total gene profile (Table 2). At 24 weeks of age, differential expression was found in 11 genes (five upregulated and six downregulated) in HCQ-treated mice (Table 3) and six genes (four upregulated and two downregulated) in GTW-treated mice (Table 3), representing 13.09% and 7.14% of the gene profile, respectively.

Discussion

SS is a common autoimmune disease with a high prevalence in the middle-aged and in older women. Reduction or complete loss of the exocrine glandular function severely diminishes the quality of life in patients with SS. Due to the unclear aetiology, the current therapies mainly focus on controlling the symptoms. Although a number of therapies have been tested to treat SS, the low efficacies limit their clinical application. The results of this experimental treatment of SS in the well-established

Table 2 Ratio of gene expression between the mice before and after disease onset.

Gene	8w/24w
GDF15	0.14
BMP10	0.20
GDF3	0.22
GDF10	0.39
IL-17f	0.45
IFNG	0.49
IL-10	0.54
CS40LG	0.61
IFNA2	0.62
BMP2	0.66
BMP7	0.68
BMP8b	0.68
IL-2	0.70
FLT3L	1.26
IL16	1.72
GDF1	2.56

Ratio < 1, gene significantly downregulated; Ratio > 1, gene significantly upregulated.

Table 3 Ratio of gene expression between the mice in the HCQ and GTW groups.

Gene	HCQ/Control	GTW/Control
GDF10	0.22	
BMP10	0.56	
IL17C	0.63	0.64
CD40LG	0.69	
FasL	0.70	
LTB	0.70	
IFNG		0.75
TNFSF4	1.58	
CSF1	1.75	
TNFSF13	1.82	
IL27	2.05	
IL1F9	4.72	3.29
IL1A		2.35
IL12B		2.44
GDF3		3.48

Ratio < 1, gene significantly downregulated; Ratio > 1, gene significantly upregulated.

NOD mouse model indicate that the traditional Chinese herb medicine GTW is effective in the protection of salivary glands by reduction in saliva secretion, morphological destruction induced by B lymphocytes and alteration in gene expression. After treatment with GTW, the stimulated whole saliva flow rate, SMG index and the Treg cell ratio were significantly higher, whereas anti-SSA/anti-SSB antibodies and the number of lymphoid foci were remarkably lower in the treated mice compared to the untreated mice. The effectiveness of the GTW treatment was similar to HCQ treatment. All of these clinical, biochemical and histological examinations suggest that GTW has the ability to protect salivary gland function, ameliorate lymphocytic infiltrations, normalise anti-SSA /–SSB profiles and recover saliva secretion. Importantly, no side effects were noted in any GTW-treated mice, whereas depilation and swelling were found in five out of nine mice in the HCQ group after the age of 16 weeks.

Some studies have shown that Treg is capable of preventing systematic autoimmunity, suppressing tumour immunity and promoting transplantation tolerance[18,19]. Unsurprisingly, the ratio of Treg cells was significantly higher in GTW- or HCQ-treated mice than in untreated mice. By considering the treatment-induced biochemical and histological changes, it is assumed that Treg cells may help generate inhibitory cytokines and suppress autoimmunity. Nonetheless, the possible role of Treg cells in preventing sialoadenitis requires further investigation. It has been proposed that the T-cell-mediated cytotoxicity and imbalance of TH1/TH2 subgroups may be responsible for the loss of salivary gland function in SS[20,21]. Some pro-inflammatory cytokines such as IL-2, IL-6 and IFN-γ (mainly TH1-type cytokines) are significantly increased in patients with SS[3-5], while the anti-inflammatory cytokines such as IL-10 and IL4 (TH2-type cytokines) are not[22,23]. TH17 is a group of T cells that may also be involved in the development of autoimmune diseases, including SS. Its effector, IL-17, has been detected in saliva and the labial glands of patients with SS[24].

Compared to the baseline, after the disease was fully developed, the gene expression profile was significantly changed. The TNF family members (TNFSF18 and TNFSF11), TH1 type cytokines (IFNG, IFNA2 and IL2), TGF-β superfamily members (GDF15, GDF10, BMP10, BMP2, BMP7 and BMP8b), TH17 type cytokine (IL-17f), CD40LG and IL-10 were significantly increased, whereas IL-16 and GDF1 decreased. TNF can enhance the IL-dependent thymocyte and T cell proliferation, and promote the production of IL-2, CSF and IFN-γ[25]. The TGF-β superfamily is active in the regulation of TH17/Treg and has dual regulatory function, which may be inclined to promote inflammation with the presence of IFN-γ[26]. CD40LG is the ligand of CD40, which is mainly expressed in $CD4^+$ T cell. Its long-term expression induces the production of autoantibody[27]. IL-10 is a TH2-type cytokine, while with the presence of IFN-γ, it tends to act as a pro-inflammatory factor[22]. After SS was fully developed in NOD mice, the expression of TH1 and TH17 cytokine genes was significantly increased while the expression of TH2 genes was slightly increased. We assumed that the T-cell-mediated immune response, the imbalance of TH1/TH2 and the increased expression of TH17 plays a critical role in the development of SS.

In HCQ-treated mice, the expression of GDF10, IL17C, LTB, FasL and CD40LG was decreased, whereas the expression of TNFSF13, CSF1, IL-27 and IL1F9 increased significantly. FasL is the ligand of Fas, which can bind with Fas, transferring the signal of apoptosis to the cell surface[28]. IL1F9, also known as IL-1 receptor 2 (IL1R2), is a molecular decoy that traps IL-1β and is not capable of imitating subsequent signalling pathways to suppress an inflammatory response[29,30]. IL-27 belongs to the IL-6/IL-12 family, which is a TH1 cytokine; however, studies have demonstrated that IL-27 is capable of suppressing the production of TH17 cells[31,32]. LTB and TNFSF both belong to the TNF family, but their expression patterns are different. The reason for this may be because the TNF family has a double function of promoting or inhibiting inflammation[33], suggesting that they may be either protective or destructive in the progression of SS. It is assumed that HCQ works mainly by suppressing the TH17-mediated immune response and the cell apoptosis process.

In GTW-treated mice, the expression of IFNG and IL17C was significantly decreased, whereas the expression of IL1A, IL12B and IL1F9 increased remarkably. The results suggest that GTW may influence the process to regulate the T-cell-mediated immune response. It may directly suppress the pro-inflammation function mediated by TH1 and TH17 cells.

In conclusion, imbalance in the TH1/TH2 system and the TH17-mediated immune reaction may play crucial roles in the development of SS-like disease in the NOD mouse model. GTW has a similar effectiveness with HCQ but with fewer side effects in the suppression of SS progression in this animal model. GTW may rescue the dysfunction of salivary glands by correcting the imbalance of pro-inflammation as well as the anti-inflammation systems and by suppressing the TH17-mediated inflammation. However, although NOD mice are well-established and widely used animal

models that can mimic SS disease, they cannot simulate the whole profile of this autoimmune disorder. Further randomised, double-blind, placebo-controlled clinical trials are needed for confirming the efficacy of GTW in the treatment of SS.

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