Original article

Effects of *Ganoderma lucidum* spores on sialoadenitis of nonobese diabetic mice

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Keywords: *nonobese diabetes; sialoadenitis; ganoderma lucidum spores*

Background Sjögren syndrome (SS) is a systematic autoimmune disease, on which traditional therapeutic agents show limited effect. More effective agents with longer-lasting and fewer side effects are needed in the clinic. The aim of this study was to investigate the effects of *Ganoderma lucindum spores* (GLS) on sialoadenitis of nonobese diabetic (NOD) mice.

Methods Thirty-two female NOD mice were assigned randomly into 4 groups: low-dose GLS-treated (L-GLS) group and high-dose GLS-treated (H-GLS) group, a dexamethasone group, and a normal saline (NS) control group. Stimulated total saliva flow rate (STFR), area of lymphocytic infiltration in submandibular glands and ratios of $CD4^+$ and $CD8^+$ T lymphocytes and B lymphocytes in peripheral blood as well as apoptosis of these subsets and serum IgG level were tested after 10 weeks of treatments. Differences among the groups were analyzed by one-way analysis of variance (ANOVA), Student-Newman-Keuls Test (SNK) was used between each two groups and a P <0.05 was considered statistically significant.

Results STFR of the high-dose GLS group increased significantly after a 10-week treatment compared with those of the NS control group (P <0.05). The incidence of sialoadenitis in GLS-treated NOD mice groups showed no significant difference compared with the control group (P >0.05), but the area of lymphocytic foci in both the H-GLS and L-GLS groups decreased significantly to 50% of the NS control group (P <0.05); the ratio of $CD4^+/CD8^+$ T lymphocytes and apoptosis of B lymphocytes of NOD mice with sialoadenitis were less and apoptosis of $CD4^+$ and $CD8^+$ T lymphocytes were significantly increased compared with the control group (P <0.05). After pretreatment with H-GLS before sialoadenitis onset, the ratio of $CD4^+/CD8^+$ T lymphocyte and the serum IgG levels of NOD mice decreased significantly (P <0.05).

Conclusions Pretreatment with H-GLS can relieve symptoms of sialoadenitis in NOD mice. GLS has some protective effects on sialoadenitis in NOD mice through increasing STFR and decreasing the area of lymphocytic foci by regulating the ratio of $CD4^+/CD8^+$ T and apoptosis of B lymphocytes.

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Sjögren syndrome (SS) is systematic autoimmune disease, lymphocytic infiltration in salivary glands leads to saliva decrease. Etiology of SS includes inhibited $CD8^+$ T cellular immunity and accentuated humoral immunity.[1-4] It has been suggested that apoptosis of T lymphocytes in peripheral blood increased because of Fas expression, while apoptosis of T lymphocytes in lymphocytes foci in salivary glands of SS patients decreased because of bcl-2 expression.[5-7] Apoptosis of B lymphocytes in peripheral blood of nonobese diabetic (NOD) mice was less than that in the control BALB/c mice.[6,8] The contradictive status of the immune cells is unexplained. Immune inhibitor adrenocortical hormones, cytotoxins of cyclosporine, hydrochloroquine, methotrexate, and the immune enhancers IFN-α and thymosin have been used to treat SS. They can relieve dry mouth slightly but have side effects at the same time, such as secondary inflammation and hypertension.[9-12] In patients treated with the anti-CD22 antibody epratuzumab, B-cell levels had been reduced by a mean of 54% and 39% after 6 and 18 weeks of treatment, but no significant change was shown on T-cell levels.[13] Recently, pilocarpine and cevemeline have been used widely for SS, they require higher doses after long time treatment, then side effects appear.[14-16] So effective therapeutic agents with long-last and fewer side effects are needed.

Traditional Chinese medicine (TCM) can regulate the balance in the bodies and have fewer side effects. Total glycosides of paeony (TGP) have been used for treatment of SS.[17] The effects of triterygium glycosides total (TGT), triterygium hypoglaucum hutch (THH), and *Celastrus aculeatus Merr*. on rheumatoid arthritis have been

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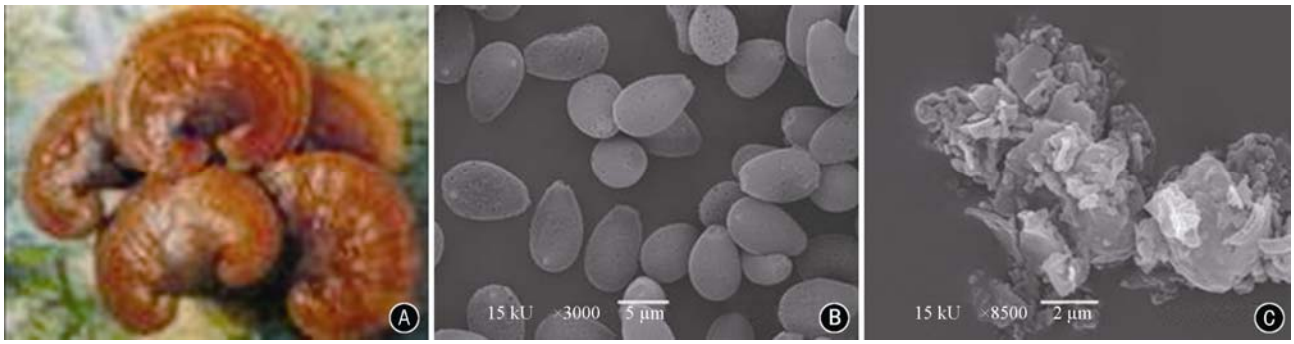

Figure. *Ganoderma lucidum* (**A**), completed *Ganoderma lucidum* spores under scan electro-microscope (**B**), and completely sporoderm-broken *Ganoderma lucidum* spores used in the test (**C**).

confirmed.[18,19] *Ganoderma lucidum* spores (GLS) have special effects on immune system regulation, which has many effective components including polysaccharides, polypeptides, triterpenes, nucleotides, amino acids, and other organic or inorganic compounds. Sporoderm-broken GLS is more easily absorbed than GLS. Modern research verified that GLS has multiple functions, such as anti-allergy effects blocking histamine release and inhibiting an over-stimulated immune system, it effects the local immunity in rheumatoid arthritis by changing the cytokines which are released by synovial fibroblasts and has an effect on regulating cellular and humoral immunity.[20-25] GLS has been mainly used in treatment of tumors,[26] its effects on SS have not been confirmed.

NOD mice are an inbred strain that develops a spontaneous sialoadenitis that is more similar to SS than any other animal model because of decreased saliva flow rate and the occurrence of auto-antibodies.[27] However, the lymphocyte subsets status of NOD mice is not clear. In this study we investigated the effects of sporoderm-broken GLS on sialoadenitis of NOD mice and explored the mechanisms of the immune regulation of lymphocyte ratios and apoptosis in peripheral blood.

METHODS

Experimental animals and treatments

The experiment protocols were admitted by the institutional ethics committee. Mice were obtained from Peking University Health Science Center Animal Care Facility and bred under special pathogen free conditions. They were assigned randomly into 4 groups: low-dose GLS-treated (L-GLS) group, high-dose GLS-treated (H-GLS) group, a dexamethasone (DEX) group, and a normal saline (NS) control group, each including 8 female NOD mice of 5-week and matched BALB/c mice. The 4 groups were treated respectively with 0.2 ml low-dose of 0.1 $g \cdot kg^{-1} \cdot d^{-1}$ completely sporoderm-broken GLS, high-dose of 1 $g \cdot kg^{-1} \cdot d^{-1}$ GLS (Figure) (Huolijian International Co. Ltd, China), 5 $mg \cdot kg^{-1} \cdot d^{-1}$ dexamethasone, and 0.2 ml of normal saline (NS) as the negative control. All mice were treated by gastric perfusion once a day for ten weeks. Saliva, sera and submandibular glands of all groups were collected at the end of the experiments.

Determination of stimulated total saliva flow rate (STFR)

After mild anesthesia with 5 mg/100 g body weight hydrochloric ketamin (Shanghai First Biochemistry Pharmaceutical Co. Ltd, China), total stimulated saliva of each mouse was collected for 15 minutes by a micropipette after stimulation with 0.05 mg/100 g body weight pilocarpine (JinYao Aminoacid Co. Ltd, China) and 0.20 mg/100 g body weight hydrochloric isoproterenol (Shanghai Hefeng Pharmaceutical Co. Ltd, China) intraperitoneally. STFR (μl/min) was counted according to the study of Hu et al.[28]

Area of lymphocytic foci in submandibular glands

The mice were sacrificed by cervical dislocation after saliva and blood collection. The submandibular glands were dissected and part of them were fixed in a 4% paraformaldehyde PBS solution (pH 7.2–7.4) and stained with hematoxylin/eosin according to the report of Robinson et al.[29] The photos were taken with a Leica 300F digital light microscope. The area of the lymphocyte foci was acquired by Leica Qwin image analysis and image processing software (Leica Microsystems Ltd, Germany).

Ratio and apoptosis of lymphocyte subsets in peripheral blood

Half of the blood of each mouse was heparin-anticoagulated, which was washed with cold PBS and about 1×10^6 cells were added into either of two eppendorf tubes. Then 1.5 μl FITC labeled anti-CD3 antibody and 3 μl APC labeled anti-CD8a antibody were added into one tube, and 2.5 μl FITC labeled anti-CD19 antibody into the other. They were incubated for 20 minutes at room temperature in the dark box. One ml hemolysin was added and incubated for 12 minutes in the dark box at room temperature then cells were washed with PBS and 100 μl binding buffer was added; 5 μl PE labeled AnnexinV was added and incubated for 15 minutes in the dark; 2 μl 7AAD was finally added and assayed by FACCalibur flow cytometry (BD PharMingen, San Diego, CA). Cells labeled with $AnnexinV^+/7AAD^-$ were counted

Table 1. Different incidence, STFR, number and area of LF of each treatment group (n=8)

Mice	NS group	DEX group	H-GLS group	L-GLS group
NOD				
Incidence	7/8†	0*	7/8†	5/8†
STFR (μg·min^{-1}·100g^{-1})	18.67±14.88†	16.83±16.58†	28.20±13.31*†	22.00±23.53†
Number of LF	9.8±5.0†	0*	3.1±0.9*†	7.6±3.5†
Area (μm^2)	37 764±24 196†	0*	18 651±10 879*†	19 340±10 434*†
BALB				
Incidence	0	0	0	0
STFR (μg·min^{-1}·100g^{-1})	43.57±13.45	26.33±13.89*	51.14±22.89	60.14±17.21*
Number of LF	0	0	0	0
Area (μm^2)	0	0	0	0

$^{*}P$ <0.05, compared with NS group of the same mice. $^{\dagger}P$ <0.05, compared with BALB/c mice. LF: lymphocytes foci.

as the apoptotic cells with the CellQuest analysis software. $CD3^+$ T%, $CD3^+CD4^+$ T%, $CD3^+CD8^+$ T%, $CD3^+CD4^+/CD3^+CD8^+$, $CD19^+$ B%, and apoptosis percentage of each subsets were recorded.

Quantification of serum IgG level

Half of the blood of each mouse was centrifuged to collect serum and kept at –20°C. Serum IgG levels were quantified with the Mouse IgG ELISA Quantitation Kit (Bethyl laboratories, INC, TX, USA). The 96-well plates were pre-coated with 100 μl of 1:100 carbonate-bicarbonate buffer diluted primary antibody from the kit at 4°C overnight; then washed 3 times. This was followed by addition of 100 μl of 1:2 serially diluted 500 ng/ml standard IgG into seven wells and 1:2000 diluted samples into other assigned wells. The plates were incubated at room temperature for 1 hour, and the wells were washed 5 times. Then 100 μl of 1:50 000 diluted horseradish peroxidase conjugated antibody was added into each well. The samples were washed 5 times again after one hour incubation at room temperature. Color was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) and terminated by H_2SO_4. IgG concentration (μg/ml) was calculated according to the standard curve measured under absorbance at 450 nm.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 10.0 was used in the test. Numerical variables were expressed as mean±standard deviation (SD). Differences among the groups were analyzed by one-way analysis of variance (ANOVA). Student-Newman-Keuls Test (SNK) was used between each two groups. A P <0.05 was considered statistically significant.

RESULTS

Determination of STFR

STFR of H-GLS treated NOD mice increased significantly after 10 weeks of treatment compared with that of the NS group (P <0.05). STFR of the DEX group did not change significantly after treatment (Table 1).

Lymphocytic foci in submandibular glands

The incidences of sialoadenitis in H-GLS and L-GLS treated NOD mice groups were respectively 7/8 and 5/8, which had no significant difference compared with that of the NS group (7/8) (P >0.05); but the area of lymphocytic foci in the H-GLS and L-GLS groups decreased significantly to about 50% of that in the NS-treated group (P <0.05), while there was no significant difference between the two GLS treated groups. No sialoadenitis was observed in the DEX treated group (Table 1).

Ratio and apoptosis of peripheral lymphocytes subsets

The ratio of $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes and apoptosis percentage of these two subsets in NOD mice was higher, while $CD19^+$ B lymphocytes apoptosis was lower than those in the BALB/c mice of the NS control group (P <0.05). The ratio of $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes and apoptositic percentage of these two subsets in H-GLS treated group was significantly lower than that in the NS group (P <0.05), while apoptosis in the $CD19^+$ B lymphocytes increased (P <0.05). Compared with the NS NOD mice group, the percentage of $CD3^+$ T and $CD19^+$ B lymphocytes in the two GLS groups did not change (P >0.05), while which in the DEX treated group decreased significantly (P <0.05) (Table 2).

Quantification of serum IgG level

The results showed that the NOD mice groups treated with H-GLS and DEX had lower serum IgG levels (P <0.05). L-GLS did not affect the IgG level of NOD mice (P >0.05), but could increase the IgG concentration in BALB/c mice (P <0.05) (Table 2).

DISCUSSION

The effects of TCM, such as TGT, TGP or *Celastrus aculeatus Merr*. on modulating the immune system or the response of immune system to antigens have been confirmed.[17-19] *Ganoderma lucidum* is a medicinal fungus with a long history in China. Modern pharmacological and clinical investigations demonstrated that *Ganoderma lucidum* has multi-activities. It has been shown that *Ganoderma lucidum* polysaccharide (GLP) has the unique ability to suppress IL-8, MCP-1 and IL-18 produced by synovia fibroblasts from rheumatoid arthritis patients.[22,30] Previous reports showed that ganopoly (a *Ganoderma lucidum* polysaccharide extract) increased the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ lymphocytes compared with baseline levels in patients with advanced-stage cancer[31] and treatment with ganopoly significantly increased cytotoxic T lymphocyte cytotoxicity and NK

Table 2. Ratio and apoptosis of peripheral lymphocytes subsets and serum IgG level (*n*=8)

Mice	NS group	DEX group	H-GLS group	L-GLS group
NOD				
$CD3^+$ T	68.81±12.57	54.79±10.64*	74.56±7.56	65.57±16.89
$CD4^+/CD8^+$	5.44±0.40†	4.76±0.23	2.83±0.69*†	2.88±0.69
$CD4^+$ T apoptosis	36.08±14.58†	34.77±4.80†	31.12±6.37*	32.62±14.13
$CD8^+$ T apoptosis	35.80±16.62†	32.28±2.38†	31.32±7.83*	10.00±3.59*
$CD19^+$ B	10.04±3.46	1.73±1.21*†	8.33±2.51	8.42±4.59
$CD19^+$ B apoptosis	2.49±1.07†	11.96±7.46*†	9.21±4.19*†	33.72±19.83
IgG (μg/ml)	200.76±38.15†	79.42±31.09*†	162.59±43.35*†	171.26±22.79†
BALB/c				
$CD3^+$ T	71.65±7.19	55.35±11.30*	71.96±11.11	62.08±9.03
$CD4^+/CD8^+$	4.11±0.88	3.06±0.60	3.79±1.06	3.50±0.67
$CD4^+$ T apoptosis	16.95±5.94	19.09±7.91	14.40±5.34	22.23±16.13
$CD8^+$ T apoptosis	19.37±3.54	10.97±5.75	14.38±6.44	21.47±12.67
$CD19^+$ B	11.72±4.45	4.66±3.59*	8.60±1.57	11.55±5.56
$CD19^+$ B apoptosis	4.80±2.01	3.86±4.21	4.60±1.90	4.60±1.04
IgG (μg/ml)	65.14±11.12	27.33±7.99*	63.24±8.77	75.16±11.74*

*P <0.05, compared with NS group of the same mice. †P <0.05, compared with control BALB/c mice.

activity in mice.[32]

In this study we investigated the effects of GLS on sialoadenitis in NOD mice, which was similar to that of patients with Sjögren syndrome. Our results showed that the ratio of $CD3^+CD4^+/CD3^+CD8^+$T lymphocytes in peripheral blood in the NOD mice group was higher than that in control BALB/c mice. It indicated that the immune status of peripheral blood of NOD mice was the same as that of the patients with SS.[33] The H-GLS treated NOD mice group had higher stimulated saliva flow rate and less area of lymphocytic infiltration compared with the NS control group. Pretreatments on NOD mice with GLS before sialoadenitis onset relieved the symptoms of salivary glands. In addition, the ratio of $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes and apoptosis of $CD4^+$ and $CD8^+$ T lymphocytes decreased after pretreating with H-GLS compared with the control group. The change in the balance of $CD3^+CD4^+/CD3^+CD8^+$ T lymphocytes relieved the sialoadenitis and could avoid inflammation secondary to treatments by other immune inhibitors. Our results showed similar effects of GLS in NOD mice with autoimmune disturbance, as ganopoly that regulated the lymphocytes in patients with advanced-stage cancer.[31] GLS regulated the immune system imbalance through modifying apoptosis of the peripheral lymphocyte subsets on different levels. In addition, H-GLS significantly decreased the serum IgG level, which is parallel with the severity of sialoadenitis in NOD mice,[34] it also increased apoptosis of B lymphocytes. The effects of GLS on apoptosis of T subsets and B lymphocytes were bidirectional, and the concentration-related effects of enhancing cellular immunity and inhibiting humoral immunity may be the mechanisms to relieve the symptoms of sialoadenitis of NOD mice. In other studies, GLS increased the number of antibody-producing lymphocytes in NIH mice and enhanced their humoral immunity.[24] In contrast, some studies showed that the extractant of GLS could inhibit the primary response of mice to sheep red blood cells and decrease the circulating antibiodies.[21] It could be deduced that GLS can also regulate humoral immunity of individuals with different immune status in bidirectional ways. The bidirectional effects of GLS are characteristics that are better than other immune inhibitors, cytotoxins and other TCMs used in autoimmune disease treatment nowadays; the latter mostly have unidirectional effects on immune system.[9-19] These characteristics give GLS fewer side effects and make it effective at the same time. G-PS at low concentrations has stronger effects on improvement of the immune system of NIH mice;[24,25] but a high concentration has more obvious effects on NOD mice. We deduced that different concentrations of GLS may have different effects on individuals of different immune status; GLS could be used not only in antitumor treatments but also for autoimmune diseases.

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