Effects of total glucosides of paenony for delaying onset of Sjogren’s syndrome: An animal study

Chun Lei Li a,1, Jing He b,1, Zhan Guo Li b, Li Wu Zheng c,**, Hong Hua a,∗

a Department of Oral Medicine and Traditional Chinese Medicine, Peking University School and Hospital of Stomatology, Beijing, China
b Department of Rheumatology and Immunology, Peking University People’s Hospital, Beijing, China
c Discipline of oral Diagnosis and Polyclinics, Faculty of Dentistry, The University of Hong Kong, Hong Kong, China

Abstract

Objective: To investigate the effectiveness of total glucosides of paenony (TGP) on Sjogren’s syndrome (SS) using non-obese diabetic (NOD) mice model.

Study design: Twenty-seven 8-week-old female NOD mice were assigned into TGP group, hydroxychloroquine (HCQ) group and normal saline (NS) group, receiving corresponding drugs respectively and sacrificed at 24-week-old. Saliva flow rate (SFR), ration of regulatory T cells, level of anti-SSA/SSB, histological changes in submandibular glands (SMG) and microarray analysis were assessed. The data were analyzed using SPSS.

Results: Compared to NS group, in TGP group, SFR, SMG index and the ration of regulatory T cells were significantly higher, while anti-SSA/SSB and lymphocytic foci were significantly lower. HCQ group demonstrated similar results except SMG index. Altered gene expression was found in 10.71% of TGP and 13.09% of HCQ of the profile.

Conclusion: TGP demonstrated a similar effectiveness as HCQ in delaying the onset of SS-like disease in NOD mice.

© 2012 European Association for Cranio-Maxillo-Facial Surgery. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Sjogren’s syndrome (SS) is a chronic disorder with focal lymphocytic infiltration in salivary and lachrymal glands leading to dry mouth and dry eye. It is one of the most common autoimmune diseases around the world. The reported prevalence varies from 0.4% to 0.7% (Vitali et al. 2002; Sardenberg et al. 2010). The aetiology-pathogenesis resulting in the loss of immune tolerance and the aggressive infiltration in exocrine glands is largely unknown. The current therapies are mainly palliative.

Hydroxychloroquine (HCQ) is used in systemic treatment of SS (Kruize et al. 1993; Tishler et al. 1999). By interfering with antigenic processes, HCQ is beneficial to SS by improving salivary gland secretion and suppressing the production of disease-associated factors including IgG, antinuclear and rheumatoid factor (Tishler et al. 1999; Dawson et al. 2005; von Bultzingslowen et al. 2007; Thanoustavraki and James 2008). However, long-term administration of HCQ may cause ocular toxic effects like outer retinal damage and pigmentary retinopathy (Tehrani et al. 2008).

Total glucosides of paenony (TGP) are the powder substances extracted from the root of Paenia lactiflora pall. Its main effective component is peoniflorin, accounting for over 90% in all substances (WU 1985). Wu et al. has reported that TGP has anti-inflammatory and antioxidative actions by suppressing the expression of intercellular adhesion molecule-1, interleukin-1, tumor necrosis factor-alpha and 3-nitrotyrosine protein (Wu et al. 2009). TGP has demonstrated its biological safety after long-term clinical use without serious side effects. Some recent studies have demonstrated the potential of TGP for treating adjuvant arthritis, collagen-induced arthritis and rheumatoid arthritis (Zhang and Wei 2005; Zhu et al. 2005; Wang et al. 2011). However, the investigation of TGP in treatment of SS is rare.

As a spontaneous model of SS, non-obese diabetic (NOD) mice have been widely used to investigate the pathological mechanism...
and management of this disease (Cha et al. 2002a; Gillespie et al. 2008; Nguyen et al. 2008). NOD mice can develop lymphocytic function causing diabetes and SS-like disease (Cha et al. 2002a,b; Jonsson et al. 2006; Jonsson et al. 2007; Chiorini et al. 2009). The present study aimed to investigate the effectiveness and safety of TGP on SS using NOD mice model by comparing to HCQ, which is used in systemic treatment of this disease with known side effects.

2. Materials and methods

2.1. Animals and treatments

Eight-week-old female NOD mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. The animals were provided with standard rodent feed and water ad libitum and housed in the Animal Unit of Health Science Center Peking University. The experiment was approved by the Institutional Ethics Committee of Peking University.

Twenty-seven NOD mice were randomly assigned into three groups, nine in each. Four or five mice from the same group were housed in one cage. From the age of 8-week to 24-week, the mice were administered with TGP (100 mg/kg/d, Ningbo Liwah Pharmaceutical Co., Ltd., China), HCQ (50 mg/kg/d, Shanghai Zhongxi Pharmaceutical (Group) Co. Ltd., China), or normal saline (NS) every other week. The mice were anesthetized with tribromoethanol and sacrificed, the submandibular glands (SMG) were dissected out within 10 min. The SMG index was obtained (weight of the submandibular glands/weight of the mice) (Wang et al. 2007). The glands were then fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were prepared and the H&E staining was performed to examine the infiltration of the tissues. Clusters of lymphocytes in a 4 mm² area are considered abnormal. The number of lymphocytic foci was counted using an irrigation stomach needle. All the mice were sacrificed at 24 weeks of age.

2.2. Salivary flow rate measurement

Stimulated total salivary flow rate (SFR) was measured every other week. The mice were anesthetized with tribromethanol (0.36 g/kg body weight, Alfa Aesar, Ward Hill, MA, USA) by intraperitoneal injection. After 5 min, the mice were given pilocarpine (0.5 mg/kg body weight, Sigma, USA) to stimulate the secretion of saliva. Saliva was collected for 15 min and the SFR was counted according to the protocol reported by Hu et al. (1992).

2.3. Autoantibody qualification

The blood was harvested from inner canthus at 10-, 16- and 24-week of age. The IgG class autoantibodies against the ribonuclear proteins SSA/Ro and SSB/La (Euroimmun, Lübeck, Germany) in serum were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacture’s protocol.

2.4. Exponent and histological analysis of submandibular glands

After the mice were sacrificed, the submandibular glands (SMG) were dissected out within 10 min. The SMG index was obtained by comparing the weight of the submandibular glands and the mice body weight (SMG index = weight of the submandibular glands/weight of the mice) (Wang et al. 2007). The glands were then fixed in 10% formalin and embedded in paraffin. Five-micrometer sections were prepared and the H&E staining was performed to examine the infiltration of the tissues. Clusters of ≥50 lymphocytes in a 4 mm² area are considered abnormal. The number of lymphocytic foci was obtained using the protocol described by Qi et al. (2009).

2.5. Flow cytometric analysis

The spleen monocytes of the mice were harvested at 24-week of age. The cells were stained with anti-CD4-FITC (BD PharMingen, San Diego, CA, USA), and then incubated and permeabilized in Fix/Perm buffer (eBioscience, San Diego, CA, USA) and stained in anti-FoxP3-PE (eBioscience). The isotype-matched control antibody was used appropriately in FACS analyses. Cells information was analyzed on a FACS Calibur flow cytometer using Cell Quest software (BD PharMingen).

2.6. Microarray analysis

At 24-week of age, three mice were randomly selected from each group and their spleen T cells were subjected to microarray analysis. Eighty-four cytokine genes related to inflammation and autoimmunization including interferon, interleukin, TGF-β family and TNF superfamily were chosen for microarray analysis.

Total RNA was extracted from the 1 × 10⁶ spleen T cells of each mouse by TRIZOL (Invitrogen life technologies, Carlsbad, CA, USA). DNase was used to remove the contaminating DNA from the RNA preparations. OD assay was measured to calculate the concentration and 1% formaldehyde-agarose gel to check the quality of RNA product. First strand cDNA synthesis and real time-polymerase chain reaction (PCR) were conducted in superarray PCR master mix (SA Biosciences, QiAGEN, Hilden, Germany). 2(ΔΔCt) method was used to analyze the data obtained from each array (Livak and Schmittgen 2001).

2.7. Statistical analysis

All the experimental data was analyzed by ANOVA using SPSS 11.5 software (SPSS² Base 11.5, Wacker Drive, Chicago, IL, USA). The statistical significance level was considered at P < 0.05.

3. Results

3.1. Determination of salivary flow rate

SFR demonstrated a declining tendency alone with the aging of the mice in all three groups. Since 10-week, mice in TGP and HCQ groups showed higher SFR than NS group (Fig. 1). There was no significant difference between TGP and HCQ group.

3.2. Quantification of serum autoantibodies

Compared to NS group, the concentrations of anti-SSA/Ro and anti-SSB/La were significantly lower in TGP and HCQ groups (Fig. 2). No significant difference was found between TGP and HCQ groups.

![Fig. 1. Stimulated total salivary flow rates of the NOD mice in the control and treatment groups at different time points. The SFR in HCQ and TGP groups are significantly higher than in NS group. *P < 0.05, TGP VS. NS group; #P < 0.05, HCQ vs. NS group.](image-url)
3.3. Submandibular glands index

The SMG index of TGP group (7.79 ± 1.10) was significantly higher than that of NS group (6.30 ± 0.96; \( P < 0.05 \)), while there was no significant difference between HCQ group (6.78 ± 0.77) and NS group at 24-week of age.

3.4. Histological examination of the submandibular glands

Inflammation was observed in all of the mice at 24-week of age. The lymphocytes were mainly found around the vessels and ducts. The structure of acinus was destroyed completely in severe infiltration area. The lymphocyte foci in TGP group (2.89 ± 1.69) and HCQ group (2.22 ± 0.83) were significantly less than that in NS group (7.33 ± 1.22, \( P < 0.05 \)). There was no significant difference between TGP and HCQ groups (Fig. 3).

3.5. Determination of the Treg in the spleen cells

The ratio of CD4+FoxP3+ T cells among CD4+ T cells was significantly higher in TGP (23.51 ± 6.38) and HCQ (20.88 ± 2.27) groups than that in NS group (15.11 ± 3.71, \( P < 0.05 \)) at 24-week of age. The two experimental groups had no significant difference.

3.6. Microarray analysis

Compared to NS group, altered expression was found in 11 genes (13.09% of the profile; five upregulated and six downregulated) in HCQ group (Table 1) and nine genes (10.71% of the profile; four upregulated and five downregulated) in TGP group (Table 2).
The mice in HCQ group showed depilation and swelling of the scalp in five of nine mice from 16- to 24-week-old. These five mice were from different cages (two from one cage and three from another). No similar symptoms were observed in the mice in TGP and NS groups during the experiment period.

### 3.7. Clinical examination

The mice in HCQ group showed depilation and swelling of the scalp in five of nine mice from 16- to 24-week-old. These five mice were from different cages (two from one cage and three from another). No similar symptoms were observed in the mice in TGP and NS groups during the experiment period.

### 4. Discussion

SS is a common autoimmune disease which may significantly compromise patients' quality of life. The etiology of SS has not been fully understood and the current therapies are mainly palliative. The present study investigated the effectiveness of a traditional Chinese medicine, TGP, in inhibiting SS-like progress using NOD mice model. HCQ, a commonly used drug for treating autoimmune diseases including SS was used as positive control.

In both TGP and HCQ groups, IL-17 family members (IL-17 in TGP group, IL-17c in HCQ group), Fasl and TGF-β superfamily members (bmp10 in TGP group, gdf10 and bmp10 in HCQ group) were downregulated, while IL-27 and IL1f9 were upregulated. Fasl is the ligand of Fas, which can bind with Fas and transfer the signal of apoptosis to the cell surface (Manganelli and Fietta 2003). The function of TGF-β is complicated. Contradictory results have shown that TGF-β was responsible for either repairing or damaging of tissues (Border and Ruooslahi 1992; Letterio and Roberts 1998; Yamano et al. 2012). Border et al. reported that over-expression of TGF-β is associated with unresolved inflammation in a lesion (Border and Ruooslahi 1992), while Yamano et al. (2012) demonstrated that decreased expression of TGF-β was associated with delayed oral wound healing. Hall et al. found that the transgenic mouse with upregulated TGF-β exhibited hypo-salivation resulted from the salivary gland fibrosis and acinar atrophy (Hall et al. 2010). IL-27 belongs to IL-6/IL-12 family, which is TH1 cytokine. Studies demonstrated that IL-27 was capable of suppressing the production of TH17 cells (Yoshimura et al. 2006; Fitzgerald et al. 2007). IL1f9, also known as IL-1 receptor 2 (IL1R2), is a molecular decoy that traps IL-1β and not capable of imitating subsequent signaling pathway so as to suppress an inflammatory response (Mantovani et al. 1998; Mantovani et al. 2003).

The expression of the TNF superfamily has different patterns in the two experimental groups. Tnfsf14 in TGP group and tnsfsf 13 in HCQ group were upregulated, while Ltb in HCQ group was downregulated. Recent studies have shown that TNF superfamily have a double function inducing either cellular proliferation, survival, and differentiation or apoptosis (Aggarwal 2003) suggesting that TNF superfamily may play either protective or destructive role in autoimmune diseases. However, the mechanism still remains unknown.

These results suggest that TGP and HCQ share some common mechanisms in suppressing TH17 mediated immune response and cell apoptosis in the treatment of SS-like disease on NOD mice. We also found that TGP and HCQ may function in different pathways in treating SS. CD40lg is the ligand of CD40 mainly expressed in the CD4+ T cell. Hernandez et al. reported that the long-term expression of CD40lg induced the production of autoantibody (San Miguel Hernandez et al. 2007). Our results showed that CD40lg was significantly downregulated in HCQ group. In TGP group, TH1 cytokine gene IFN-r and TH2 cytokine gene IL-10 were downregulated, indicating that TGP directly suppressed the pro-inflammation function mediated by TH1 cytokines.

Recent researches have demonstrated that regularly T cell may play a crucial role in transplantation tolerance, preventing autoimmunity and controlling tumor immunity (Lund et al. 2008; Sakaguchi et al. 2008). To investigate the difference of Treg in the control and experimental groups, the ratio of CD4+FoxP3+ T cells among CD4+ T cells in the mice spleen cells was examined in the

---

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>HCQ/NS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>gdf10</td>
<td>0.22</td>
<td>0.00</td>
</tr>
<tr>
<td>bmp10</td>
<td>0.56</td>
<td>0.04</td>
</tr>
<tr>
<td>il17c</td>
<td>0.63</td>
<td>0.01</td>
</tr>
<tr>
<td>cd40lg</td>
<td>0.69</td>
<td>0.01</td>
</tr>
<tr>
<td>fasl</td>
<td>0.70</td>
<td>0.01</td>
</tr>
<tr>
<td>ltb</td>
<td>0.70</td>
<td>0.00</td>
</tr>
<tr>
<td>tnf5f4</td>
<td>1.58</td>
<td>0.02</td>
</tr>
<tr>
<td>csi1</td>
<td>1.75</td>
<td>0.04</td>
</tr>
<tr>
<td>tnf5f13</td>
<td>1.82</td>
<td>0.048</td>
</tr>
<tr>
<td>il27</td>
<td>2.05</td>
<td>0.00</td>
</tr>
<tr>
<td>il1f9</td>
<td>4.72</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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

**Table 2**

<table>
<thead>
<tr>
<th>Gene</th>
<th>TGP/NS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>bmp10</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>il17f</td>
<td>0.19</td>
<td>0.00</td>
</tr>
<tr>
<td>il10</td>
<td>0.27</td>
<td>0.00</td>
</tr>
<tr>
<td>ltb</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td>fasl</td>
<td>0.33</td>
<td>0.00</td>
</tr>
<tr>
<td>il27</td>
<td>1.36</td>
<td>0.03</td>
</tr>
<tr>
<td>tnf5f14</td>
<td>3.94</td>
<td>0.02</td>
</tr>
<tr>
<td>il1b</td>
<td>4.93</td>
<td>0.04</td>
</tr>
<tr>
<td>il1f9</td>
<td>18.23</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Ratio <1, gene significantly downregulated; ratio >1, gene significantly upregulated.
present study. The significantly higher ratio of Treg in the treatment groups, together with the significantly lower titer of the autoantibodies and less destruction of the glands indicating that Tregs may help to generate inhibitory cytokines and suppress autoimmunity. Some reports showed that IL-10 was crucial in the peripheral generation of Tregs (Wan and Flavell 2006; Hong et al. 2009), while other studies demonstrated that Treg cells were unable to produce or respond to IL-10 suggesting that IL-10 may not be essential for the function of Treg (Dieckmann et al. 2001; Joneilet et al. 2004).

In this study, we did not find a positive correlation between IL-10 and Treg. The differences in experimental model, methodology and the multifunction of Treg may contribute to the contradictory results. In summary, we demonstrated that TGP has a similar effect to HCQ but with fewer side effects in ameliorating lymphocytic infiltrations, normalizing autoantibody profiles and slowing the decrease of saliva secretion in a NOD mouse model. TGP may delay the onset of SS by suppressing TH1 and TH17 mediated inflammation process in NOD mice. We also noted that SFR changed before the autoantibodies profile; it is assumed that TGP may have early and direct effectiveness on the multifunction of Treg before the autoantibodies profile. Therefore, although NOD mice are good animal models which can mimic SS-like disease, it cannot simulate the whole profile of this autoimmune disorder. Further randomized controlled clinical trial is necessary before we can offer solid therapeutic recommendations of using TGP for treating SS.

5. Conclusions
The pathogenesis of SS remains unknown, and the current therapeutic procedures mainly focus on relieving the symptoms. TGP demonstrated similar effectiveness to HCQ in delaying the onset of SS-like disease in NOD mice. Although HCQ has been commonly used for treating autoimmune diseases, it is an effective but not highly potent medication for managing SS. TGP will not provide a final solution but may be a safe alternative in the management of SS.

Conflict of interest
None of the authors claim a conflict of interest or funding.

Acknowledgment
This study was supported by National Natural Science Foundation of China (Grant Nos. 30672320 and 31070788). We gratefully thank stuff from Peking University Science Center Animal Care Facility for their technical support.

References