Full Length Research Paper

Therapeutic effect of a Chinese herbal compound on spontaneous lupus MRL/lpr mice

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This study aimed to assess the therapeutic effects of Wu-Di-Tang (WDT) on lupus prone MRL/lpr mice and provide an experimental basis for clinical applications. Thirty-two female MRL/lpr mice were randomly assigned to treatment and control groups, 16 in each group. Oral administration of WDT and saline to treated and control mice began from 12 weeks of age and continued for 16 weeks. The efficacy of WDT was evaluated by assessing survival rate, lymphadenopathy, C-reactive protein (CRP), proteinuria, serum creatinine and dsDNA autoantibody in continuous samples taken at different time points (12, 16, 20, 24 and 28 weeks), and pathological changes of the kidneys at 28 weeks. WDT effectively prolonged the survival of lupus mice, inhibited the production of serum anti-dsDNA autoantibody (p < 0.05, 0.01), reduced lymph node enlargement (p < 0.05, 0.01, 0.001) and serum CRP (p < 0.01), decreased the levels of proteinuria and serum creatinine (p < 0.05, 0.01, 0.001), and ameliorated the pathology of lupoid nephritis in treated mice compared to control animals. These data indicate that WDT has the potential to regulate the immune system, inhibit inflammatory reactions, improve kidney function, and ultimately produce beneficial effects on Systemic lupus erythematosus (SLE) mice. Moreover, it is promising for WDT to be developed as a novel immunotherapeutic agent to treat SLE.

Key words: Systemic lupus erythematosus, Wu-Di-Tang (WDT), Chinese herbal compound, MRL/lpr mice.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies that can form immune complexes which are deposited in tissues, leading to inflammation and irreversible damage to organs including the kidneys, lung and brain (Balsamo and dos Santos-Neto, 2011; La Cava and Lourenco, 2009). Conventional and typical treatment approaches include the use of immunosuppressive medications (corticosteroids and chemotherapeutic agents), non-steroidal anti-inflammatory drugs (NSAIDs), and antimalarial drugs (Bernknopf et al., 2011). Unfortunately, current treatment options are often insufficiently efficacious, high cost, or poorly tolerated (Li et al., 2009; Schmuk and Yazdany, 2011). As a result, SLE patients often seek complementary therapies (Chou, 2010).

The use of Chinese herbal compound (CHC) has generated abundant practical experience in this field, and good clinical effects have been achieved (Yap et al., 1999; Zhang et al., 2011). According to the theory of traditional Chinese medicine (TCM), the major pathogenesis of SLE is a
common deficiency complicated with excessiveness, and the combination of reinforcing deficiency and reducing excess is the basic treatment principle for SLE (Yang and Xu, 2005). Until now, however, the English literature suggest that no study is concerned about the above two sides simultaneously.

Wu-Di-Tang (WDT), composed mainly of Zhi-Bai-Di-Huang-Wan and Qin-Hao-Bie-Jia-Tang, two famous Chinese medicine recipes, acts equally on the deficiency and the excess, and is widely used for the treatment of lupus erythematosus in our clinic. Therefore, it felt necessary to carry out a systematic pharmacological study to provide an experimental basis for clinical application of WDT.

Animal models are extremely useful tools in defining the pathogenesis and improving treatment of human disease. MRL/lpr mice are considered to be an excellent and spontaneous model of SLE (Perry et al., 2011). These mice develop high-titer anti-DNA antibodies and immune complex-mediated nephritis and exhibit progressive lymphadenopathy, which are similar to those seen in human lupus erythematosus. MRL/lpr mice have been widely utilized as an animal model for studying the pathogenesis and evaluating the treatment effect of drugs (Rottman and Willis, 2010).

In the present study, autoimmune-prone female MRL/lpr mice were used to validate whether WDT has beneficial effects on the autoimmune disease and explore its mechanism from the perspective of immune regulation, inflammation inhibition, and renal protection function.

**MATERIALS AND METHODS**

**Preparation of WDT extract**

The formulation of WDT consists of twelve different medicinal plant ingredients as shown in Table 1. These were purchased from Shanghai LeiYunShang Pharmaceutical Co., Ltd (Shanghai, China). The authenticity of all these crude drugs was confirmed by Dr. M. Y. Wang (School of Pharmacy, Shanghai Jiaotong University, Shanghai, China) using histological techniques. To prepare the aqueous extract of WDT, 200 g of dried material was extracted with 1 L of boiling water for 2 h, and then the supernatant of the first extraction was removed. The same procedure was repeated two times. The decoction obtained from the three extractions was combined, filtered, and concentrated. Finally, the filtrate was prepared in the form of freeze-dried powders for experimental use.

**Experimental animals**

Thirty two female ten-week-old MRL/lpr mice were purchased from the Scientific Animal and Plant Center of Fudan University (Shanghai, China). Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed, as well as the current version of the China Law on the Protection of Animals. The mice are known to be SLE model mice, which spontaneously develop SLE-like manifestations. Mice were housed in well-ventilated rooms (at 19 to 23°C, and 45 to 70% relative humidity) with a regular light-dark cycle (12 h of light, 07:00 to 19:00 h), and food and tap water were provided ad libitum. At 12 weeks of age, the animals were randomly divided into two groups, with 16 mice in each group. Treated mice were given an oral clinical equivalent dose of WDT (5 g/kg body weight/day) and control mice were administered the same volumes of saline; both groups were treated from the age of 12 to 28 weeks.

**Evaluation of the survival rate and lymphadenopathy of MRL/lpr mice**

The date of any deaths among the mice was recorded and the percentage of living mice was calculated. In addition, the diameter of each palpable lymph node was measured every 4 weeks in millimeters (mm). The sum of the individual lymph node scores was assigned to each mouse as a total score.

**Urine collection and proteinuria assay**

Every 4 weeks, from the age of 12 to 28 weeks, 24 h urine from individual mice was collected using metabolic cages. Proteinuria was tested using the Coomassie brilliant blue test (Mo et al., 2010), using albumin (bovine serum) to make the standard curves. Briefly, murine urine was centrifuged at 3000 rpm for 10 min. The supernatant was diluted at 1:10 with distilled water, and optical density was measured at 595 nm after addition of Coomassie brilliant blue solution.

**Assays for serum ds-DNA and C reaction protein**

For the detection of specific antibodies in the sera of immunized mice, serum ds-DNA and C-reactive protein (CRP) were analyzed using Enzyme-linked immunosorbent assay (ELISA) kits purchased from Alpha Diagnostic International Inc (San Antonio, TX) and R&D Systems Inc (Minneapolis, MN). All ELISAs were performed according to the manufacturer’s instructions. Briefly, 100 µl of each of the diluted serum samples, as well as the negative and positive controls, were added to the wells of a plastic microplate pre-coated with dsDNA. The plate was then incubated for 30 min at room temperature (approximately 23°C). After washing three times with the washing buffer, 100 µl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was added to each well and the plate was incubated for a further 30 min. After incubation, the wells were washed three times and 100 µl of a given substrate solution

**Table 1. Composition of Wu-Di-Tang (WDT).**

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rehmannia glutinosa</em> (Gaertn.) Libosch</td>
<td>30</td>
</tr>
<tr>
<td><em>Cornus officinalis</em> Sieb. et Zucc.</td>
<td>15</td>
</tr>
<tr>
<td><em>Paeonia suffruticosa</em> Andr.</td>
<td>15</td>
</tr>
<tr>
<td><em>Anemarrhena asphodeloides</em> Bge</td>
<td>15</td>
</tr>
<tr>
<td><em>Phellodendron amurense</em> Rupr</td>
<td>20</td>
</tr>
<tr>
<td><em>Alisma orientale</em> (Sam.) Juzep.</td>
<td>10</td>
</tr>
<tr>
<td><em>Artemisia apiacea</em> Hance</td>
<td>15</td>
</tr>
<tr>
<td><em>Amyda sinensis</em> (Wicgmann)</td>
<td>10</td>
</tr>
<tr>
<td><em>Polygonum multiflorum</em> Thuab</td>
<td>30</td>
</tr>
<tr>
<td><em>Polygonatum sibiricum</em> Redoute</td>
<td>15</td>
</tr>
<tr>
<td><em>Paeonia lactiflora</em> Pall</td>
<td>30</td>
</tr>
<tr>
<td><em>Viola yedoensis</em> Mak.</td>
<td>20</td>
</tr>
</tbody>
</table>
Age (weeks)  
Lymphatica (mm)  
Survival rate  

Figure 1. The survival rate and progression of lymphadenopathy in MRL/lpr mice. The survival rate represents the percentage of mice surviving at weekly timepoints. Mice treated with WDT showed a higher survival rate than those in the control group (A). The progression of lymphadenopathy was delayed by WDT treatment. A significant difference was observed after 4 weeks of therapy (B). Results are expressed as the mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001, WDT-treated mice versus control mice.

Evaluation of kidney function

The animals were bled by retro-orbital puncture every 4 weeks, and serum samples were obtained by centrifugation (1500 rpm for 5 min). Serum samples were stored at -80 °C until use. The serum was used to monitor creatinine levels, which was quantitatively determined colorimetrically using picrate as described by Jaffe (Mali and Nicholas, 1988).

Kidney pathology

Mice surviving to 28 weeks of age were sacrificed and kidneys were removed for histopathological analysis. The freshly harvested right kidney of each mouse was fixed in buffered 10% formalin for at least 72 h, and embedded in paraffin blocks. Seven-micron-thick sections were cut and stained either with hematoxylin and eosin, or with periodic acid-Schiff (PAS) using standard procedures. Sections were blindly graded semi-quantitatively by one pathologist for glomerular, interstitial and vascular lesions, and PAS positive deposition according to the previously described method (Kon et al., 2008; Shlomchik et al., 1999). Scores from 0 to 4 (where 0 is no damage and 4 = severe) were assigned for each of these features.

The other kidney from each mouse was snap-frozen in optimal cutting temperature compound. To detect immune complex (C3) deposits, cryostat sections (2 μm) were fixed in chilled acetone and stained with primary antibody C3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and then stained with a fluorescein isothiocyanate (FITC)-conjugated goat polyclonal anti-mouse IgG (Jackson Immunoresearch laboratories Inc., West grove, PA). For negative controls, sections were treated with normal goat C3. Immunofluorescent-stained slides were read in a blinded fashion and graded 0 to 3, according to the following criteria: 0 = no staining; 1 = mild staining; 2 = moderate staining; and 3 = high staining (Tomlinson et al., 2008).

Statistical analyses

Data were analyzed using Student’s unpaired t-test if they were normally distributed (Kolmogorov–Smirnov test). Otherwise, the Mann–Whitney U-test was used. All statistical analyses were performed using statistical package for social sciences (SPSS) 16.0 and a two-tailed value of p < 0.05 was considered significant.

RESULTS

Effect of WDT on survival rate of MRL/lpr mice

To determine whether the oral administration of WDT could alter the survival time of MRL/lpr mice, the survival rate was monitored as shown in Figure 1A. Among the control mice, the first death was observed at week 21, and only 70% survived to 28 weeks of age. In contrast, the first death among the mice treated with WDT was observed on week 24, and 90% survived to 28 weeks of age. This shows that the survival of mice administered WDT was significantly prolonged in comparison with that of the control mice.

Effect of WDT treatment on lymphadenopathy and C reactive protein

MRL/lpr mice are also characterized by pronounced lymphadenopathy, especially in the neck. The degree of lymphadenopathy was compared between the control group and the treated group. WDT-treated mice had significantly smaller lymph nodes than control mice (Figure 1B). In most cases, the lymph node score of
Effect of WDT treatment on anti-ds-DNA

The serum anti-dsDNA autoantibody titer data are presented in Figure 2B. According to data from the control group, anti-dsDNA autoantibody increased over time until it peaked at 20 weeks of age, after which a slight decrease was observed. In comparison with the control group, the serum mean titers of anti-dsDNA autoantibody in treated mice paralleled those in controls and no significant difference between groups was observed before 20 weeks. In the treated group, however, values were significantly reduced during the later stages of the illness, and a significant difference was present at 24 and 28 weeks (p < 0.05).

Effect of WDT treatment on serum creatinine and proteinuria

At 12 weeks, no significant differences in serum creatinine or proteinuria concentrations were found between the two groups. With regard to the serum creatinine levels, the control group showed a progressive increase, while the treated group seemed to have a tendency to decrease from the age of 16 weeks. A statistically significant difference was observed at age 20 weeks (p < 0.05), which was maintained at 24 and 28 weeks (p < 0.001) (Figure 3A). In addition, the change in proteinuria in both groups of animals was in the shape of a sinusoidal curve (Figure 3B). The proteinuria levels significantly decreased during the early phase from 12 to 20 weeks, but this was followed by an increase after the age of 20 weeks. However, mice treated with WDT had significantly lower proteinuria levels than control mice at all time-points tested from 16 weeks (p < 0.001 at 16 and 20 weeks, p < 0.01 at 24 weeks, p < 0.05 at 28 weeks).

Effect of WDT treatment on kidney pathology and C₃ immune complex deposition

The renal histology shown in Figure 4 illustrates the obvious histopathologic changes that occur in lupus nephritis. All kidneys from both groups had glomerular, interstitial and vascular lesions and the glomeruli were the most severely affected. Most mice treated with WDT had less severe kidney disease than controls, with significantly diminished glomerular and perivascular lesions (Figure 4A and B). C₃ staining was observed only in glomeruli, and the intensity of C₃ correlated with the severity of proteinuria (Figure 4C). Remarkable deposition of C₃ was observed in the kidneys of all mice in the control group. In contrast, significantly less glomerular C₃ deposition was noted in the mice treated with WDT. Figure 4D shows the results of renal lesion scores in the two groups. Glomerular, interstitial, and vascular lesions, and C₃ positive deposition were significantly less severe in the mice treated with WDT than in those treated with treated mice was only half that of control group mice at the same time-point, and a statistically significant suppression was observed after 4 weeks of therapy with WDT. The severity of lupus correlates with the degree of systemic inflammation which is mirrored by the level of CRP in the plasma. During the observation period, from 12 to 24 weeks, CRP levels remained stable in both groups. During the last month, CRP levels of the treatment group mice dramatically decreased, while they significantly increased in the control group, and a significant difference was observed (p < 0.05) (Figure 2A).
Serum creatinine (µg/L) 24 h proteinuria (mg/24 h) Age (weeks)

Figure 3. Serum creatinine and proteinuria in MRL/lpr mice. Treatment of MRL/lpr mice with WDT attenuated the elevation in both serum creatinine and proteinuria levels seen in control animals. (A) Shows the significant difference in serum creatinine levels between controls and WDT-treated animals at 20, 24 and 28 weeks, (B) shows the marked reduction in proteinuria observed in WDT-treated mice at 16, 20, 24 and 28 weeks. WDT-treated mice versus control mice, *p < 0.05, ***p < 0.01, ****p < 0.001.

with saline. The most significant differences between groups were in glomerular lesions (p < 0.05), vascular lesions (p < 0.01) and C3 immunocomplex deposition (p < 0.01).

DISCUSSION

From the theory of traditional Chinese medicine, the pathogenesis of SLE is commonly thought of as a mixed deficiency-excess condition, which belongs to the kidney yin deficiency, blood stasis and heat toxin excess type, and the basic therapy is to reinforce deficiency and reduce excess. The deficiency is treated by nourishing yin to reduce fire, and the excess is treated by activating blood and clearing heat and toxins (Yang and Xu, 2005). According to English literature, several studies focusing on reinforcing deficiency have been reported, such as Liu-Wei or Ba-Wei-Di-Huang-Wan (Chen et al., 2011; Furuya et al., 2002), Ren-Shen-Yang-Rong-Tang (Kawakita et al., 1998; Nakai et al., 1993; Zhou et al., 1994), and Chi-Shie-Shuang-Bu-An-Shen-Tang (Wu et al., 2007). The herbs used in these formulations, called the "superior herbs" in Chinese medicine, are used to reinforce deficiency, fortify the body and mind, and also enhance adaptability to stress.

On the other hand, the method of reducing excess has also been studied for its ameliorating effects on SLE in recent years. Long-Dan-Xie-Gan-Tang is a representative recipe for eliminating heat and purging fire, and has dramatic immunomodulatory action in MRL-mediated immune dysfunction and kidney injury (Chang and Lee, 2010). In this study, the approach of reinforcing deficiency coupled with reducing excess was applied to investigate its effects on the treatment of lupus-prone MRL/lpr mice. WDT includes 12 types of herbs and is mainly based on Zhi-Bai-Di-Huang-Wan and Qin-Hao-Bie-Jia-Tang, two famous traditional Chinese medicine recipes. Di-Huang-Wan has been shown to be an effective prescription for the amelioration of severe SLE in both humans and mice (Chen et al., 2011; Furuya et al., 2002). Qin-Hao is the main herb in Qin-Hao-Bie-Jia-Tang; moreover, artemisia annua is the active component of Qin-Hao, which has also been verified to be beneficial in SLE (Sun and Zhang, 2009). Therefore, WDT conforms to the principle of TCM on SLE, and many of the herbs chosen have been proven to be effective for the treatment of SLE.

Consequently, in the present study, a dramatic prolongation of survival was observed in mice treated with WDT compared to control animals (Figure 1A). We may be able to find a bigger difference or a statistical significant difference if we can observe the experimental animals till their death due to natural causes. The result indicates that WDT is effective in suppressing the progression of disease and has beneficial effects on SLE.

SLE, a systemic autoimmune disorder, is characterized by the formation of autoantibodies against DNA structures. Autoantibodies can initiate the inflammatory cascade, trigger cellular damage and cause glomerular disease due to the deposition of immune complexes in the kidney (Stohl and Jacob, 2010). Anti-dsDNA autoantibodies play a critical role in the pathogenesis of SLE. After 8 weeks continuous administration of WDT, the serum anti-dsDNA level was significantly decreased in treated mice compared with controls (Figure 2B). This suggests that WDT administration inhibits the production of autoantibody and regulates the autoimmune response.
Figure 4. Representative images and renal lesion scores in MRL/lpr mice. (A) HE staining, (B) PAS staining, (C) immunofluorescent staining of kidney sections. Glomerular lesions, interstitial and perivascular disease were all more severe in the control group than in the WDT-treated group. Less inflammatory cell infiltration in the kidneys of the WDT-treated animals was noted (A, B). Glomerular deposition of the complement component C3 was significantly reduced in mice treated with WDT (C). Glomerular and perivascular lesions and C3 deposition were all significantly less severe in WDT-treated mice than in control mice (D). Results are expressed as the mean ± SD; *p < 0.05, **p < 0.01, WDT-treated mice vs. control mice. Scale bars represent 50 µm.

MRL/lpr is also characterized by spontaneous and remarkable lymphoproliferation at the body surface (Bobé et al., 2006). Therefore, by using autoimmune MRL/lpr mice, we were able to directly observe the inflammatory reaction by measuring the enlargement of the lymph nodes. In addition, CRP is the prototypical acute phase serum protein, rising rapidly in response to inflammation. The serum CRP level is usually used in the clinic as an important index of inflammatory severity (Hilgers and Stumpf, 2009). Thus we can indirectly assess the inflammatory response by detecting CRP.

In this study, lymph node enlargement was obviously inhibited after 4 weeks of WDT treatment (Figure 1B). Meanwhile, a significant reduction of CRP levels was also observed in the last 4 weeks in the WDT treated mice (Figure 2A). As shown above, a direct anti-inflammatory effect has been demonstrated by the finding that WDT can inhibit lymphadenopathy, and an indirect anti-inflammatory effect has been shown by the differences in CRP level between the two groups in the final 4 weeks.

SLE is a multi-organ disease, and renal involvement affects up to 60% of patients. Lupus nephritis (LN) is a major clinical problem because of its high morbidity and mortality rates. Proteinuria is the presence of protein in the urine, which is often an early indication of kidney disease (Contreras et al., 2010). Creatinine is a waste product of creatine phosphate metabolism by skeletal muscle tissue and is freely filtered by the renal glomerulus. Renal dysfunction diminishes the ability to filter creatinine and consequently serum creatinine rises. Therefore, the serum creatinine level has been found to be a fairly reliable indicator of kidney function. In our study, the serum creatinine level gradually increased with advancing age in control group mice (Figure 3A). Conversely, serum creatinine seemed to have a tendency to decrease in the treated group.

A similar kinetic was observed when proteinuria was measured in consecutive urine samples (Figure 3B). In order to further evaluate the therapeutic consequences of WDT treatment, the findings were assessed histologically. Renal pathology in SLE involves both immune complexes and a cellular inflammatory response (Schwartz, 2007). Histology indicated that control animals exhibited classical features of glomerular disease with diffuse glomerulonephritis including cellular proliferation, inflammation (HE staining), expansion of the mesangial matrix (PAS staining) and dense C3 deposition (C3 IF). However, a significant improvement in pathological outcomes was
observed in treated animals (Figure 4). WDT treatment markedly ameliorated the glomerular injury and PAS positive deposition, as well as the formation of vascular lesions. These beneficial changes are mainly attributable to WDT inhibiting autoantibody production and immune inflammatory response, ultimately alleviating the deterioration of renal function and pathology.

**Conclusion**

In this study, we investigated therapeutic effect of WDT on MRL/lpr mice and explored its mechanism in dynamic progress of disease. The overall evidence suggests that WDT has beneficial effects on SLE and is promising to be developed as a novel immunotherapeutic agent to treat autoimmune diseases. Regulation immune system, inhibition inflammatory reaction and improvement kidney function may be its potential mechanism. This study also provided an experimental basis for WDT clinical application to SLE patients.

**ACKNOWLEDGMENTS**

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