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Effect of cyclophosphamide on fungal infection in SLE mice detected by fluorescent quantitative PCR

Hong-Fen Ge[1,2,3], Guan-Zhi Chen[3*], Min Pan[3], Hui Guo[4]

[1]*Shandong Provincial Hospital for Skin Diseases, Shandong University, Jinan 250022, Shandong, China*

[2]*Shandong Provincial Institute of Dermatology and Venereology, Shandong Academy of Medical Sciences, Jinan 250022, Shandong, China*

[3]*Department of Dermatology, The Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China*

[4]*Laboratory Department, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China*

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ABSTRACT

Objective: To observe the effect of the cyclophosphamide (CTX) on systemic lupus erythematosus mice by fluorescent quantitative polymerase chain reaction.
Methods: (BALB/c × C57BL/J6) F1 hybrid mice were used as recipients of donor lymphocytes which were injected intravenously. Mice were randomly divided into control group and treatment group, serum autoantibody and urinary protein were measured, renal pathological changes and the situation of fungal infection were observed.
Results: The reduced urinary protein, ameliorated renal lesions were found in the CTX-treated mice. *Candida albicans* and *Aspergillus flavus* were detected respectively in respiratory tract and gastrointestinal tract.
Conclusions: CTX can alleviate lupus nephritis and the fluorescent quantitative PCR is a rapid and sensitive method for the detection of fungal infection.

1. Introduction

The systemic lupus erythematosus (SLE) is an autoimmune disease that is mediated by autoimmune and is characterized by the multi-system lesions [1]. Its pathogenesis has not been clear. To further explore its pathogenesis, the appropriate animal model will be required. In this study, relying on the recognized induced SLE mouse model [2], we observed serum autoantibody, urinary protein and renal pathological changes [2–4] and determine the effect of cyclophosphamide. In addition, the fluorescent quantitative PCR [5] method was used for the detection of fungal infection of SLE model mice.

2. Materials and methods

2.1. Materials

Female BALB/c mice aged 6–8 wk and male C57BL/J6 mice aged 6–8 wk were purchased from Laboratory Animal Center of Chinese Academy of Sciences; the anti-dsDNA from Beijing H&J NovoMed Co., Ltd, goat anti mouse FITC-IgG from Sigma and DNA extraction kit and Fluorescent Quantitative PCR kit from Takara Bio. The primer probe was synthesized by Shanghai Invitrogen Biotechnology Co., Ltd.

2.2. Methods

2.2.1. Grouping of laboratory animals

The (female BALB/c × male C57BL/J6) F1 hybrid mice (BCF1) were chosen as the research subjects. Female BCF1 mice were in the weight of (18 ± 4) g. A total of 72 mice were randomly chosen for SLE induction. Another 24 mice were selected as the normal control group.

2.2.2. Induction of SLE mouse model

The lymph node, thymus and spleen of BALB/c mice were separated under sterile conditions, with the proportion of 1:2:3.

*Corresponding author: Guan-Zhi Chen, Department of Dermatology, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong, China.
Tel: +86 18661807818
Fax: +86 532 82911215
E-mail: 18661807818@163.com

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After being diluted with the normal saline, they were ground with 200 μm and 80 μm nylon sieve. The survival of lymphocytes was observed and the number of cells was counted under the microscope. The 50×10^6 living lymphocytes were injected into each female BCF1 mouse through the tail vein every time. The injection time was at 0, 3, 7 and 10 h. Mice in the normal control group were given the injection of normal saline by the equal volume.

2.2.3. Cyclophosphamide (CTX) treatment

A total of 12 wk after the modeling, 36 mice were randomly selected as the CTX administration group. The intragastrical administration was given every other day, lasting for 12 wk. The administration dose was calculated according to the following equation [6]: $d_B = d_A \times R_B/R_A \times (W_A/W_B)^{1/3}$, where, d_B, d_A are the administration dose per kilogram for animals of B and A; R_B, R_A are the body mass indices for these two animals respectively, $R_{mouse} = 90$, $R_{human} = 100$; W_A, W_B are the weight (kg) for them respectively. In addition, other 36 modeled mice were selected as the model group. Mice in the normal control group and model group were injected with the normal saline by the equal volume.

2.3. Sampling and detection

2.3.1. Analysis of anti-dsDNA antibody in serum of mice

The intravenous blood sampling was performed from the eye orbit of mice in the normal control group, model group and CTX administration group at 12th week (successful modeling), 18th week (6 wk of CTX treatment) and 24th week (12 wk of CTX treatment). The indirect immunofluorescent assay was employed to detect the autoantibody dsDNA.

2.3.2. Detection of severity of proteinuria in mice

The urine were collected using the reflex urination method from mice in the normal control group, model group and CTX administration group at 12th week (successful modeling), 18th week (6 wk of CTX treatment) and 24th week (12 wk of CTX treatment) [6]. The coomassie blue staining was employed to detect the concentration of urinary protein.

2.3.3. Pathological section staining of mouse kidney tissue

Six mice in the normal control group and model group were executed at 12th week respectively; while remained ones in the normal control group, model group and CTX administration group were executed at 24th week. The kidney was collected for the pathological section HE staining. The change in the histomorphology of kidney was analyzed under the optical microscope [7].

2.3.4. Observation of immune complexes deposited in kidney tissue of mouse

After the deparaffinage of above paraffin sections, the goat anti mouse FITC-IgG was added for the incubation. After being washed and mounted, they were observed under the fluorescence microscope for the deposition of immune complexes.

2.3.5. Ultrastructural changes of kidney tissue of mouse

The kidney tissue was fixed with the glutaraldehyde solution. After being dehydrated, primarily stained and counter stained, the ultrastructure of kidney tissue was observed under the transmission electron microscope.

2.3.6. Detection of Candida albicans (C. albicans) and Aspergillus flavus (A. flavus) by fluorescent quantitative PCR

Before the execution of mice at 24th week, the whole blood, urine and excrement of mice were collected. Only the lungs were collected from the executed mice. DNA of excrement, whole blood, urine and lung tissue was extracted respectively and then stored at −80 °C.

The DNA gene sequence of *C. albicans* and *A. flavus* could be referred to NCBI website and then the PCR primers of *C. albicans* and *A. flavus* could be designed. The sequence for 2 pairs of primers was: *C. albicans*: F 5′-TCTCCCTCAAACCGCTGGG; R 5′-GGTTAGACCTAAGCCATTGTCAAAG; *A. flavus*: F 5′-GCTGCCCATCAAGCACGG; R 5′-CCTACAGAGCGGGTGACAAAG. The 50 μL PCR reaction system contained 10 μL reaction buffer, 1 μL Taq Mix, 1 μL dNTP, and 0.5 μL upstream primer, downstream primer and probe of *C. albicans* and *A. flavus* respectively. Five μL extracted DNA was supplemented with water to 50 μL. Roche 480 PCR system was employed for the amplification reaction, with the reaction conditions as follows: 93 °C for 3 min; 94 °C for 45 s, 55 °C for 1 min, with 4–5 repeats. The fluorescence signal was collected at 55 °C.

2.4. Statistical analysis

All data was treated with SPSS13.0 for the statistical analysis, while $P < 0.05$ indicated the significant difference.

3. Results

3.1. General conditions

Modeled mice showed the reduced activity, increased weight, darkened hair and edema to different extents since the 4th week. During the period from 18th week to 24th week, 2 mice in the model group died. After the treatment, mice in CTX treatment group had the gradually increased weight, good activity and normal feeding. The conditions for mice in the normal control group were good.

3.2. Change in level of anti-dsDNA antibody in serum of mice

After the successful modeling, the titer of anti-dsDNA antibody was obviously increased in the model group and CTX administration group. At different weeks, the titer of anti-dsDNA antibody in the serum of mice that were treated with CTX was significantly lower than the one in the model group ($P < 0.05$), as shown in Table 1.

Table 1
Change in level of anti-dsDNA antibody in the SLE mice at different weeks.

Group	12th week (successful modeling)	18th week (6 wk of CTX treatment)	24th week (12 wk of CTX treatment)
Normal control group	–	–	–
Model group	11.13 ± 3.95	13.25 ± 2.11	14.15 ± 3.42
CTX administration group	10.98 ± 2.56	8.44 ± 2.01	5.24 ± 1.11

3.3. Quantitative comparison of urinary protein among mice during 24 h

After the successful modeling, mice in the model group and CTX administration group all had the proteinuria. At different weeks, the level of proteinuria for mice in CTX treatment group was significantly decreased ($P < 0.05$), as shown in Table 2.

Table 2
Change in level of proteinuria in the SLE mice at different weeks (mg).

Group	12th week (successful modeling)	18th week (6 wk of CTX treatment)	24th week (12 wk of CTX treatment)
Normal control group	–	–	–
Model group	5.68 ± 0.33	6.75 ± 0.45	7.22 ± 0.22
CTX administration group	5.54 ± 0.56	2.84 ± 0.24	0.67 ± 0.11

3.4. Pathological change of kidney tissue

The HE-stained kidney tissue of modeled mouse was observed under the microscope. The volume compensation of glomerulus was increased, as well as the number of glomus cells. The inflammatory cell infiltration and hyperplasia of endothelial cells and mesangial matrix in mice treated with CTX were improved to the different extent (Figure 1).

3.5. Deposition of immune complexes in kidney tissue

The obvious granular fluorescence could be found in the glomerulus of SLE mice. The fluorescence was deposited in the

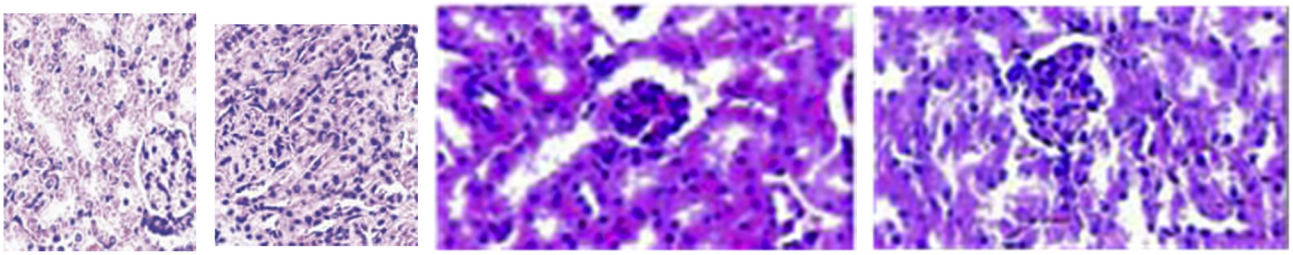

Figure 1. Pathological change of kidney tissue of SLE mouse.

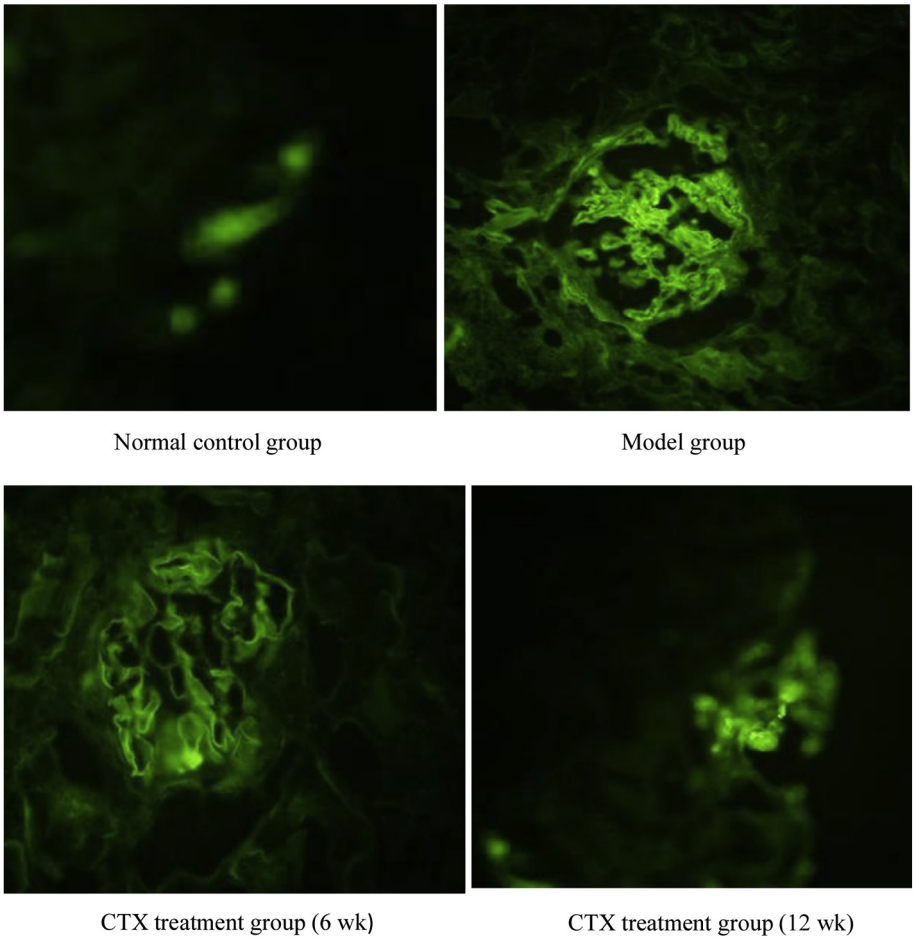

Figure 2. Deposition of immune complexes in kidney tissue.

capillaries of glomerulus. CTX-treated mice were improved to the different extent (Figure 2).

3.6. Ultrastructural change of kidney tissue

For the observation under the electron microscope, it could be indicated that the hyperplasia of endothelial cells and mesangial matrix in mice treated with CTX were improved to the different extent (Figure 3).

3.7. Defection of C. albicans and A. flavus

The fluorescent quantitative PCR was employed to detect the gene sequence of *C. albicans* and *A. flavus*. In CTX treatment group, the *C. albicans* was found in 2 mice and *A. flavus* in 1 mouse, with the detection part of lung and gastrointestinal tract. The detection rat was 6.52%. There was no fungus found in the mice in normal control group and model group.

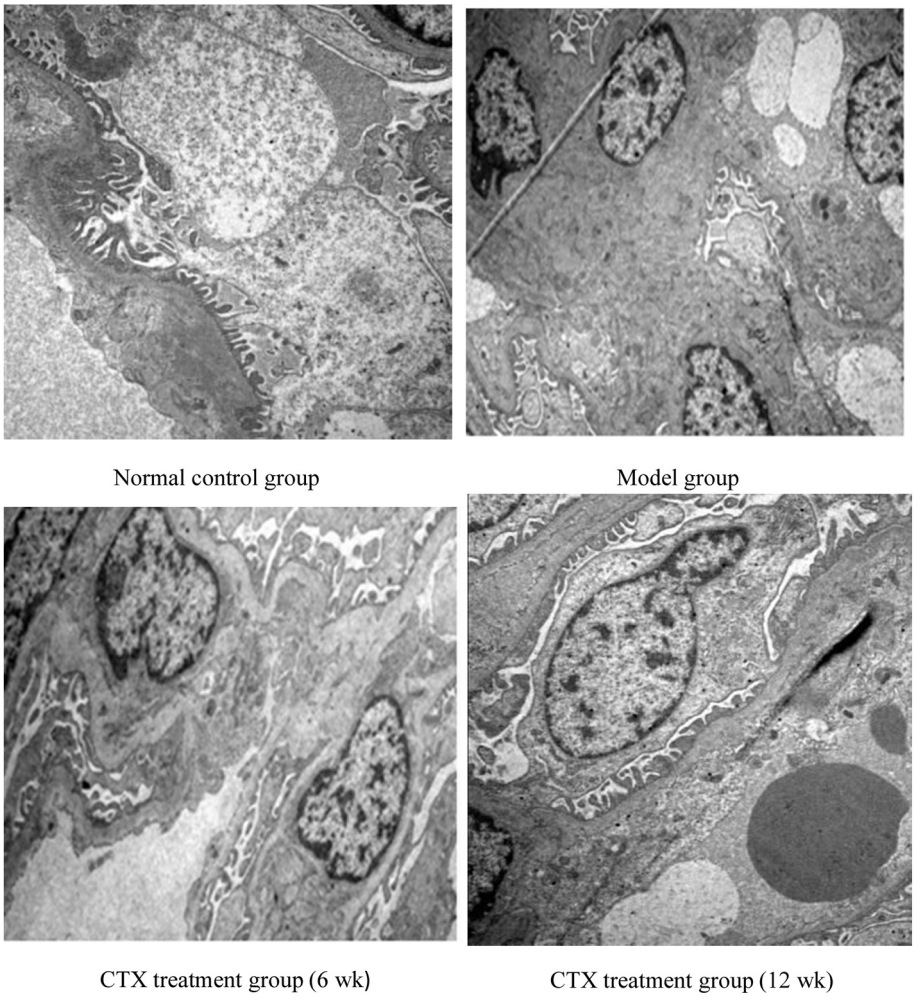

Figure 3. Ultrastructural change of kidney tissue under electron microscope.

4. Discussion

The systemic lupus erythematosus is the typical representative of autoimmune diseases. Its pathogenetic characteristic is that T and B-lymphocytes are highly activated and the level of multiple autoantibodies is extremely increased to form the high level of immune complexes and thus result in the injuries of tissues and organs [8,9]. Its pathogenesis has not been fully understood and the researches on such disease were mainly based on the animal model. According to previous findings [10,11], such model was related to the gender and it preferred to the female mice. Accordingly, (BALB/c × C57BL/J6) F1 hybrid mice were selected for the modeling. Because of the abundant source, such mouse is suitable for all kinds of laboratory researches. The disease characteristic of such SLE mouse model is the lymphoid hyperplasia to cause the autoantibody similar with SLE patients and thus result in the kidney diseases mediated by immune complexes.

Results of this study showed that, the expression of anti-dsDNA antibody was positive in induced SLE mice that had the proteinuria, which was consistent with the findings by the foreign scholar [2]. Bruijn *et al*. [12] reported that, after 12–14 wk of induction, the kidney of mice had the defused hyperplasia of glomerulus and membranous glomerulonephritis, while the mice with severe condition had the ball-like glomerulosclerosis. According to our induced model, such lesion appeared at the 12th week, mainly the abundant inflammatory cell infiltration and hyperplasia of endothelial cells and mesangial matrix. After being treated with the large dose of immunosuppressive agent, the level of anti-dsDNA antibody and proteinuria in modeled

mice were all significantly decreased. Meanwhile, the pathological change of kidney tissue had been significantly improved.

According to the clinical researches in recent years, the incidence of systemic lupus erythematosus combined with fungal infection showed the increase trend and became one of important death causes for patients with systemic lupus erythematosus [13]. The fungal infection was most common in the death because of a single pathogen infection [14]. In this study, it was the first time that the fluorescent quantitative PCR method was employed for the amplification of *C. albicans* and *A. flavus* that were commonly accompanied with the systemic lupus erythematosus. Besides, the *C. albicans* and *A. flavus* were found in the lung and gastrointestinal tract of CTX-treated mice. It was similar with the findings of Du [15] in the clinical practice that patients with systemic lupus erythematosus usually had the invasive fungal infection in the lung. It was also in accordance with the findings of Deng [16] that the pathogen for patients with systemic lupus erythematosus combined with invasive fungal infection was mainly *C. albicans*. It should be specially emphasized that the real-time fluorescent quantitative PCR method has the good sensitivity and specificity, which can overcome the disadvantages of low culture sensitivity and long culture time of fungus and thus serve as a rapid and correct method for fungus detection.

However, there are certain limitations in this study. Only the most common *C. albicans* and *A. flavus* were amplified in the laboratory, without the detection on other funguses. With the deeper understanding of systemic lupus erythematosus combined with fungal infection, the further detection on other funguses should be performed, which will lead to the better understanding of the systemic lupus erythematosus.

Conflict of interest statement

We declare that we have no conflict of interest.

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