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Clinical Study

Double filtration plasmapheresis benefits myasthenia gravis patients through an immunomodulatory action

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ABSTRACT

Double filtration plasmapheresis (DFPP) is used to treat myasthenia gravis (MG). However, the definite mechanism is unclear. This study investigated whether DFPP improves MG through an immunomodulatory action. Thirty-five MG patients were randomly divided into two treatment groups: Group A (DFPP combined with oral methylprednisolone) and Group B (oral methylprednisolone alone). Their antibody levels, clinical scores, cytokine levels, and $CD4^+CD25^{high}Foxp3^+$ (regulatory T cell [Treg]) levels were then determined. Anti-titin antibody levels were significantly lower in Group A compared with Group B after treatment. The clinical remission rate in Group A was significantly higher than in Group B. The changes in cytokine levels (interleukin [IL]-2, IL-4, IL-10, and interferon-γ) in sera and the peripheral blood mononuclear cell culture supernatants did not significantly differ before and after the treatments in both groups ($p < 0.05$). The soluble intercellular adhesion molecule-1 (sICAM-1) levels were lower in Group A than in Group B ($p < 0.05$). MG patients exhibited a lower percentage of Treg cells than normal patients. DFPP combined with methylprednisolone treatment increased the Treg cell percentage more than treatment with methylprednisolone alone ($p < 0.05$). DFPP treatment more effectively lowers sICAM-1 and increases Treg cell expression, consequently benefiting MG patients.

1. Introduction

Myasthenia gravis (MG) is a common antibody-mediated autoimmune disorder of the neuromuscular junction and its incidence has been increasing of late. The pathogenesis of MG is unclear, but circulating antibodies, cytokines, and abnormal immune activation have all been suggested to play a role in MG pathogenesis [1,2].

Autoimmune antibodies against acetylcholine receptors (AchR), presynaptic membrane (PrsmR), and titin reportedly compromise neuromuscular transmission, which leads to skeletal muscle weakness [3,4]. T and B lymphocytes, as well as cytokines, participate in the development of MG [5–7]. $CD4^+CD25^{high}Foxp3^+$ regulatory T (Treg) cells play a critical role in the regulation of immune responses in MG patients [8]. MG patients have higher levels of many cytokines, including interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ, than healthy individuals [9]. Plasma exchange has been widely applied to treat MG in clinical practice for more than 30 years because it removes pathogenic antibodies and rapidly ameliorates the associated symptoms [10]. Double filtration plasmapheresis (DFPP) is the currently favored method of plasma exchange because of its safety. Previous studies have demonstrated that plasma exchange regulates cytokine levels in MG patients [9,11]; however, few studies have shown that DFPP modulates cellular immunity [12]. We investigated the effect of DFPP on Th1 cytokines (IL-2 and IFN-γ), Th2 cytokines (IL-4 and IL-10), soluble intercellular adhesion molecule-1 (sICAM-1), and Treg cell percentages in MG patients. We determined the quantitative disease scores, as well as the amounts of anti-AchR antibody, anti-PrsmR antibody, anti-titin antibody, and cytokines before and after DFPP treatment in MG patients.

2. Subjects and methods

2.1. Subjects

Thirty-five late-onset MG patients recruited in 2010 from Huashan Hospital, Shanghai, China, were divided into two groups: Group A, in which patients were treated with a combination of

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DFPP and oral methylprednisolone; and Group B, in which patients were treated with oral methylprednisolone alone. Group A consisted of 20 patients, 11 men and nine women, with an average age of 53.2 ± standard deviation (SD) 2.3 years and pretreatment quantitative MG disease (QMG) scores [13] of 20.4 ± SD 1.7. Group B consisted of 15 patients, nine men and six women, with an average age of 54.1 ± SD 3.1 years and pretreatment QMG scores of 16.5 ± SD 1.1 (Table 1). Both groups received oral methylprednisolone (0.8 mg/kg/day), and the observation period was 14 days for each group. A total of 20 healthy age-matched and sex-matched volunteers, 10 men and 10 women, with an average age of 50.2 ± SD 1.8 years, were recruited as the control group. This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Huashan Hospital. Written informed consent was obtained from all participants.

2.2. DFPP treatment

DFPP was performed three times within 1 week using an apheresis monitor (KM 8900, Kuraray, Osaka, Japan) and blood access was obtained by inserting a double lumen catheter into the right internal jugular vein. The volume of plasma exchanged during every treatment (range: 2500–3000 ml) was dependent on patient body weight (40–50 ml/kg). Heparin was used for anticoagulation at a bolus dose of 20 mg and at 10 mg/hour for maintenance. The primary plasma separator was Plasmaflo PS-08, and the secondary plasma fractionator was a Cascadeflo EC-20W (both Kuraray) with an albumin sieving coefficient of 0.62 and albumin loss of approximately 45 grams per treatment. Therefore, 500 ml of 10% albumin solution was administered intravenously as replacement fluid (Fig. 1).

2.3. Sample collection and processing

Blood samples (10 ml) were collected from the Group B subjects and the healthy volunteers on day 1 and day 14 of the experimental treatment to determine the levels of cytokines and autoimmune antibodies. In Group A, blood samples were collected before and after each DFPP procedure, and on day 14. The anti-AchR antibody levels were measured using the AchR antibody kit (RSR, Cardiff, UK) following the manufacturer's instructions. The anti-titin antibody levels were measured by enzyme-linked immunosorbent assay (ELISA) using purified 30 kDa MG/thymoma-specific antigens (MGT-30). The MGT-30 protein, produced using an *Escherichia coli* vector, is a 30 kDa peptide that represents the main immunogenic region of the titin protein and is used to detect anti-titin antibodies following previously described methods [14]. β-Bungarotoxin–binding protein, purified from a bovine crude protein extract using affinity chromatography, was used as an antigen to detect anti-PrsmR antibody following the methods described by Lu et al. [15]. Optical density (OD) was measured at 450 nm. Sample sera and negative control sera were analyzed in the same microtiter plates. Data are expressed as the ratio (positive/negative value) between the sample OD and the negative control OD. IL-2, IL-4, IL-10, IFN-γ, and sICAM-1 in the plasma were measured using ELISA (R&D, Minneapolis, MN, USA), following the manufacturer's instructions.

Table 1
Features of 35 myasthenia gravis (MG) patients included in this study

	Group A	Group B	*p* value
Sex, male/female	11/9	9/6	>0.05
Age, years	53.2 ± 2.3	54.1 ± 3.1	>0.05
Age at onset of MG	51.8 ± 2.1	54.5 ± 3.5	>0.05
Thymoma/Thymectomy	13/7	7/8	>0.05
Clinical grade[a]			
II A	7	6	>0.05
II B	6	5	>0.05
III	7	4	>0.05
Pre-QMG score	20.4 ± 1.7	16.5 ± 1.1	>0.05

[a] Based on Osserman's classification and QMG score.
QMG = quantitative myasthenia gravis score, Pre-QMG = quantitative myasthenia gravis score before treatment.

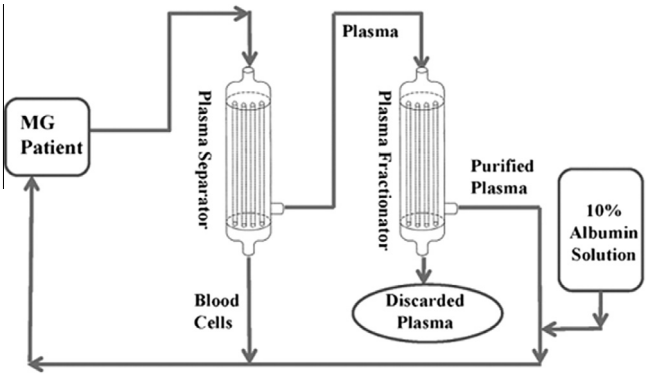

Fig. 1. Diagram of the double filtration plasmapheresis procedure. MG = myasthenia gravis.

2.4. Culture of peripheral blood mononuclear cells

Whole blood (10 ml) was collected from healthy volunteers and MG patients before and after DFPP or methylprednisolone therapy. Blood was diluted 1:1 with sterile saline. Peripheral blood mononuclear cells (MNC) were collected via density gradient centrifugation on a Ficoll–Hypaque (Sigma-Aldrich, St Louis, MO, USA) density gradient. The MNC were washed three times with 10 ml normal saline and suspended in 10 ml fetal calf serum medium (Sijiqing, Hangzhou, China). The cells were then incubated in complete medium for 48 hours at 37 °C under a 5% CO_2 atmosphere. The supernatant was collected for cytokine analysis as described in the previous section.

2.5. Flow cytometry

MNC were immunostained with fluorescein isothiocyanate or phycoerythrin (PE)-conjugated CD4 and CD25 antibodies (BD Biosciences, San Diego, CA, USA). The gates were set so that the $CD4^{+-}$ $CD25^{-}$ population was based on the isotype control whereas the $CD25^{high}$ population was determined relative to the low intensity of CD25 staining. Intracellular Foxp3 staining was performed using Foxp3 PE-Cytosine5 (PE-Cy5) tests in 1 × permeabilization buffer (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Flow cytometry was performed using a Coulter Epics XL flow cytometer (Becton Dickinson, Mountain View, CA, USA) as previously described [16]. The cells were gated based on forward-scatter and side-scatter signals to exclude dead cells and debris and establish the lymphocyte gate. Fluorescence signals were collected in log mode. Treg cell percentage was calculated by Treg/$CD4^{+}CD25^{+}$T cells.

2.6. Clinical evaluation

The severity of MG was evaluated by the same MG specialist before and after treatment using the QMG score. The QMG score is comprised of 13 parameters: time of diplopia onset, blepharoptosis, strength to close lips, ability to swallow, ability to vocalize,

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