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Original Article

Therapeutic effects of curcumin on experimental autoimmune myasthenia gravis

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Abstract: Experimental autoimmune myasthenia gravis (EAMG) is a B-cell-mediated and T-cell-dependent autoimmune disease of the neuromuscular junction in which the nicotinic acetylcholine receptor (AchR) acts as an autoantigen. Curcumin is a yellow acidic phenolic substance extracted from the rhizome of the plant turmeric. It has various pharmacological properties, including anti-oxidant, anti-cancer, and anti-inflammatory effects. However, the immunoregulatory effects of curcumin on MG and EAMG are still unclear. Hence, this study aimed to investigate the effects and potential mechanisms of curcumin on EAMG. Female Lewis rats were randomly divided into three groups: CFA group (n = 9), EAMG group (n = 8), and curcumin group (n = 7). It was found that curcumin can ameliorate clinical symptoms of EAMG rats and protect AchR from being destroyed in the neuromuscular junction (NMJ) of EAMG rats. Moreover, it was confirmed that curcumin can regulate the balance of Th1/Th2/Th17/Treg in spleens of EAMG rats. In conclusion, this study shows that curcumin can ameliorate EAMG by regulating the balance of Th1, Th2, Th17, and Treg.

Keywords: Experimental autoimmune myasthenia gravis, curcumin, Th1/Th2/Th17/Treg

Introduction

A classic model for myasthenia gravis (MG), experimental autoimmune myasthenia gravis (EAMG) is a B-cell-mediated and T-cell-dependent autoimmune disease of the neuromuscular junction in which the nicotinic acetylcholine receptor (AchR) acts as an autoantigen [1]. Since various factors are involved in the pathogenesis of MG, the etiology of MG remains unclear. It has been reported that the balance of T helper type 1 (Th1), Th2, Th17, and regulatory T (Treg) subsets of CD4+ helper T-cells are redistributed during the development of EAMG [2].

Curcumin is a yellow acidic phenolic substance extracted from the rhizome of the plant turmeric. It has various pharmacological properties, including anti-oxidant, anti-cancer, and anti-inflammatory effects [3]. Moreover, many studies have shown that curcumin can inhibit inflammation in a variety of autoimmune and inflammatory diseases, such as rheumatoid arthritis(RA) [4], inflammatory bowel disease (IBD) [5, 6], Alzheimer's disease (AD) [7], experimental autoimmune encephalomyelitis (EAE) [8], and experimental autoimmune neuritis (EAN) [9].

However, the immunoregulatory effects of curcumin on MG and EAMG remain unclear. Based on previous studies, this study aimed to investigate the effects and potential mechanisms of curcumin on EAMG and provide a new therapeutic strategy for treatment of human MG.

Materials and methods

Animals

Female Lewis rats (6-8 weeks, 160-180 g; Vital River, Beijing, China) were housed under a

12-hour light and 12-hour dark cycle with free access to food and water. Ethical approval was granted by the Ethics Committee of Wenzhou Medical University. All experimental procedures were in accordance with Care and Use of Laboratory Animals guidelines published by the China National Institute of Health. Effort was made to minimize the number of animals used and to minimize suffering.

Induction and clinical scoring of EAMG

EAMG rats were immunized by subcutaneous injections into the tails with 200 μl inocula containing 50 μg R97-116 peptides (DGDFAIVKFTKVLLDYTGHI) (AC Scientific, Inc, Xian, China) in 100 μl complete Freund's adjuvant (CFA; Sigma-Aldrich, USA), supplemented with 1 mg M. tuberculosis (Difco, Detroit, MI, USA) and 100μl phosphate-buffered saline (PBS) on day 0. Next, the rats were boosted on day 30 with the same peptide in incomplete Freund's adjuvant (IFA; Sigma-Aldrich, USA). Control rats, designated as the CFA group, received the same emulsion except PBS was used instead of the peptide.

After the first immunization, each rat was weighed and monitored for clinical scores every other day until sacrificed (6-8 weeks). The severity of clinical signs was scored by measuring muscular weakness. Clinical scoring was based on the presence of tremors, hunched posture, muscle weakness, and fatigability. Fatigability was evaluated after exercise (repetitive paw grips on the cage grid) for 30 seconds. Disease severity was expressed as follows [10]: Grade 0 = normal muscle strength; Grade 1 = mildly decreased activity, weak grip, fatigable; Grade 2 = weakness, hunched posture at rest, decreased body weight, tremors; Grade 3 = severe generalized weakness, marked decrease in body weight, moribund; Grade 4 = death. Rats with intermediate signs were assigned grades of 0.5, 1.5, 2.5, or 3.5, respectively. Results are expressed as the mean score of each group at each time-point.

Curcumin administration

Rats were randomly divided into three groups: CFA group (n = 9), EAMG group (n = 8), and curcumin group (n = 7). For treatment with curcumin (Sigma-Aldrich, USA), rats in the curcumin group were injected intraperitoneally (i.p.) with 100 mg/kg curcumin dissolved in dimethyl sulfoxide (DMSO; Vetec, Sigma-Aldrich, USA), every other day from day 0 to day 56. Rats in the CFA and EAMG groups were injected with the same volume of DMSO. All rats were weighed and examined for clinical scores every other day until sacrificed.

Flow cytometry

For detection of intracellular cytokine synthesis and expression of extracellular molecules, flow cytometry (FACS) analysis was performed, as described previously [11], with several modifications. Mononuclear cells (MNCs), isolated from spleens of rats, were incubated with Brefeldin A (1:1000 dilution, eBioscience Inc., San Diego, CA, USA) and PMA (50 ng/ml, eBioscience Inc., San Diego, CA, USA) for 4 hours at 37°C. Next, T-cells were incubated extracellularly with FITC-anti-rat-CD4 (eBioscience, San Diego, USA) for 30 minutes at 4°C while Tregs were identified by positive staining with APC-anti-rat-CD25 (eBioscience, San Diego, USA). After fixation and permeabilization, intracytoplasmic staining was carried out using one of the following fluorescently labeled Abs: eFluor660-anti-rat-IFN-γ (eBioscience, San Diego, USA), PE-anti-rat-IL-4 (BD Biosciences, USA), PE-anti-rat-Foxp3 (eBioscience, San Diego, USA), and PE-anti-rat-IL-17 (eBioscience, San Diego, USA). Isotype-matched FITC-, PE-, and APC-conjugated mAbs of irrelevant specificity were used as negative controls. The samples were detected by a flow cytometer (FACS Calibur; BD Biosciences, USA).

Immunofluorescence

Gastrocnemius muscle tissues of rats from the CFA group, EAMG group, and curcumin group were sectioned (6 μm) and incubated with pancreatic enzymes for 30 minutes. Afterward, 5% BSA was added and incubated at 37°C for 30 minutes. Next, the sections were incubated at 4°C overnight with Anti-Synaptophysin antibody (Abcam, Cambridge, MA, USA). The following day, the sections were washed with PBS-T and then incubated with FITC-labeled anti-rabbit IgG and Alexa Fluor 555-labeled-α-bungarotoxin (BTX, Invitrogen, Carlsbad, CA) at 37°C for another 1 hour. Finally, the sections were observed by fluorescent microscopy (Nikon, Japan) after staining with DAPI for 7 minutes.

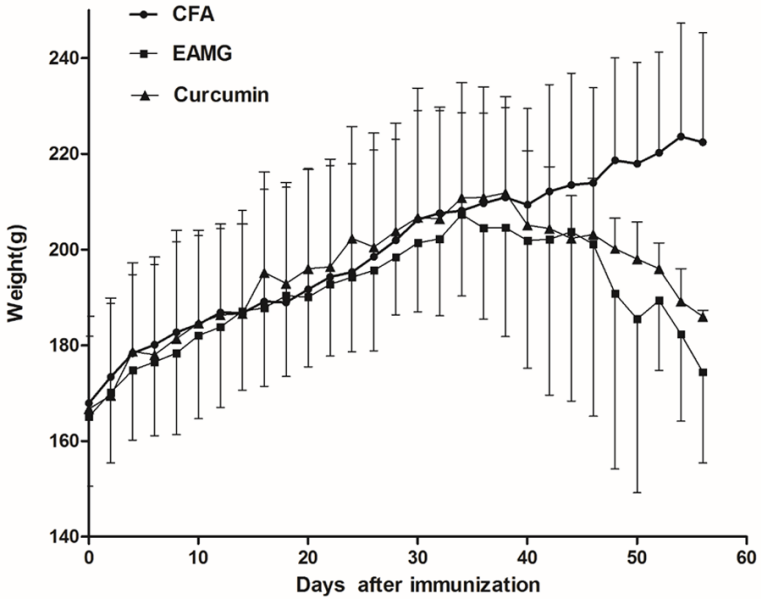

Figure 1. Effects of curcumin on body weights of EAMG rats. CFA: normal control group; EAMG: model group; Curcumin: curcumin treatment group.

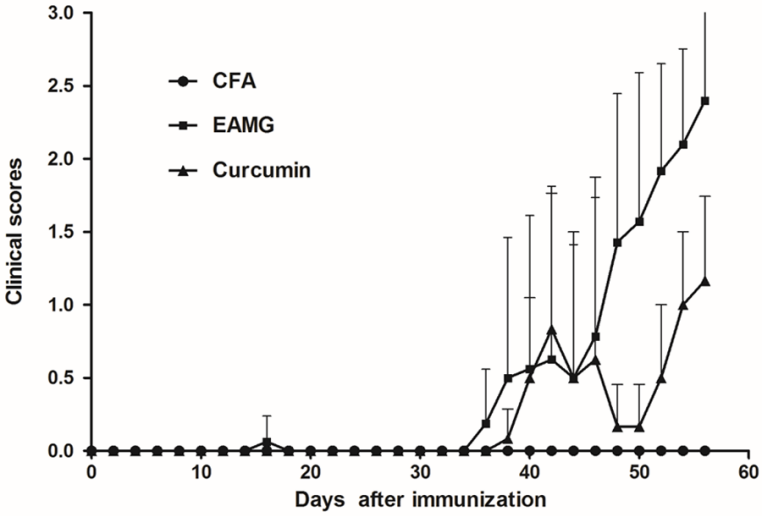

Figure 2. Effects of curcumin on clinical scores of EAMG rats. CFA: normal control group; EAMG: model group; Curcumin: curcumin treatment group.

Statistical analysis

Data was analyzed using SPSS21.0 software (SPSS Inc, Chicago, USA). Data are expressed as mean ± standard deviation (SD). Differences between the three groups were tested by single factor analysis of variance (One-Way AVOVA) followed by the least significant difference (LSD) test as a post hoc test. Level of significance was set as $p < 0.05$.

Results

Curcumin ameliorates the clinical symptoms of EAMG rats

Weight loss was less in the curcumin group, compared with the EAMG group, but there were no statistical differences between the weights of the two groups (**Figure 1**). Compared to the EAMG group (0.91 ± 1.07), the average clinical score of the curcumin group was (0.17 ± 0.29) on the 48th day, with statistically significant differences ($P < 0.05$). Differences between the average clinical score of the two groups were statistically significant from days 48 to 52 (**Figure 2**). These data indicate that curcumin can ameliorate the clinical symptoms of EAMG rats.

Curcumin protects AchR from being destroyed in the neuromuscular junction (NMJ) of EAMG rats

Using a fluorescence microscope, a certain number of nerve and AchR in NMJ in the muscles of the CFA group were observed, while the number of AchR was significantly reduced in NMJ in the muscles of the EAMG group. However, it was found that the number of AchR in NMJ in the muscles of the curcumin group was significantly increased compared to that in the EAMG group. This result suggests that curcumin, to some extent, protects AchR from being destroyed in the NMJ of EAMG rats (**Figure 3**).

Curcumin regulates the balance of Th1/Th2/Th17/Treg in spleens of EAMG rats

Compared to the CFA control group, ratios of Th1 cells (CFA: 2.66 ± 2.22, EAMG: 3.73 ± 4.09,

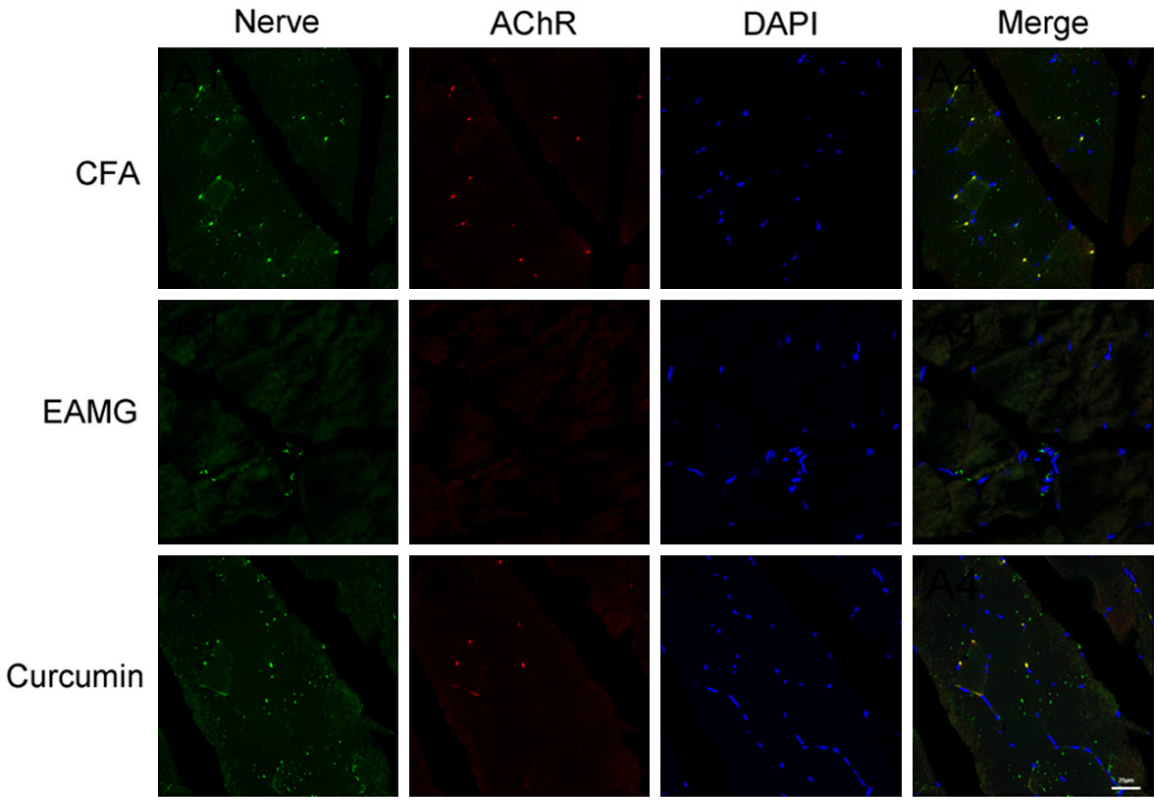

Figure 3. Paraffin sections of rat gastrocnemius muscles Immunofluorescence technique for detection of neuroendocrine (NMJ) nerve (Nerve) and AchR morphology and changes. CFA: normal control group; EAMG: model group; Curcumin: curcumin treatment group. The nerve endings (green, left) of NMJ were labeled with rabbit anti-Synaptophysin primary antibody (1:50) and FITC-labeled goat anti-rabbit secondary antibody (1:500); with Alexa Fluor555 (1:200) labeled AchR (red, left) at NMJ; cells labeled with DAPI (Blue, left three).

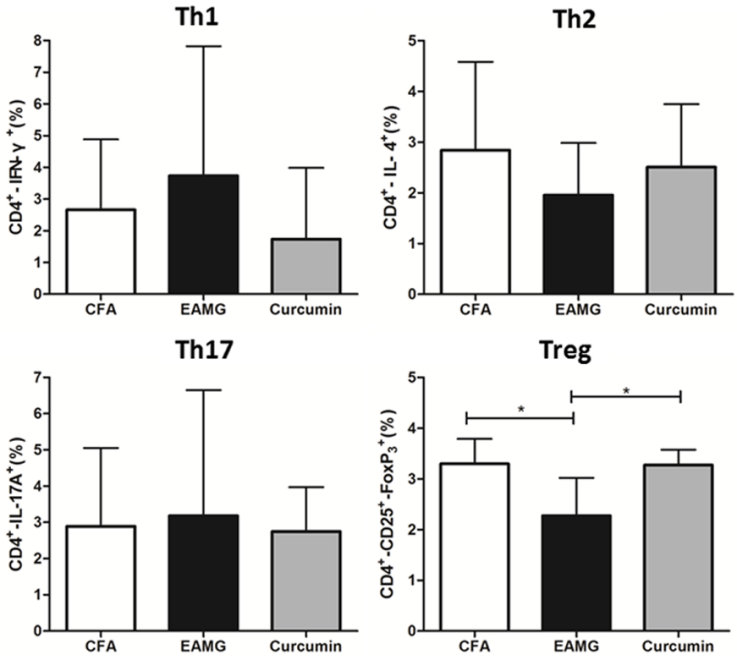

Figure 4. Percentage of CD4+ T-cells in spleens of rats. CFA: normal control group; EAMG: model group; Curcumin: curcumin treatment group. *P < 0.05.

P>0.05) and Th17 cells (CFA: 2.89 ± 2.17, EAMG: 3.18 ± 3.47, P>0.05) in the CD4+ T-cell subsets of spleens in the EAMG group were increased while the ratios of Th2 cells (CFA: 2.84 ± 1.74, EAMG: 1.95 ± 1.03, P>0.05) and Treg cells (CFA: 3.30 ± 0.49, EAMG: 2.28 ± 0.74, P = 0.018) were decreased. However, differences of Th1, Th2, and Th17 cells between the two groups were not statistically significant (**Figures 4**, **5**). Compared to the EAMG group, ratios of Th1 cells (EAMG: 3.73 ± 4.09, Curcumin: 1.74 ± 2.25, P>0.05) and Th17 cells (EAMG: 3.18 ± 3.47, Curcumin: 2.74 ± 1.23, P>0.05) in the CD4+ T-cell subsets of spleens in the curcumin group were decreased while the ratios of Th2 cells (EAMG: 1.95 ± 1.03, Curcumin: 2.51 ± 1.24,

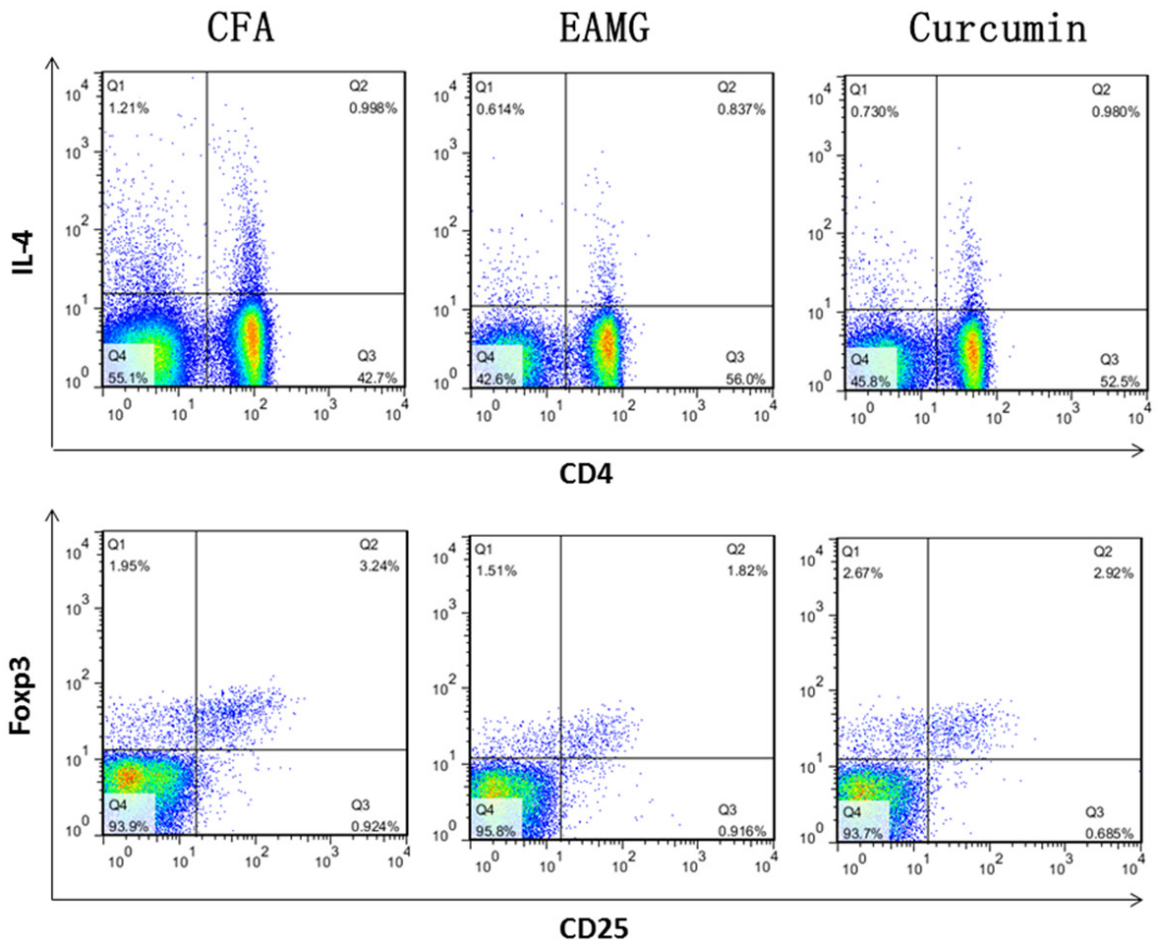

Figure 5. Proportion of Th2 (CD4+ - IL-4+) and Treg (CD4+ - CD25+ - FOXP3+) in spleens of the three groups. CFA: normal control group; EAMG: model group; Curcumin: curcumin treatment group.

P>0.05) and Treg cells (EAMG: 2.28 ± 0.74, Curcumin: 3.27 ± 0.30, P = 0.018) were increased. However, there were no significant differences between the two groups regarding Th1, Th2, and Th17 cells (**Figures 4**, **5**). Results suggest that curcumin can regulate the balance of Th1/Th2/Th17/Treg in spleens of EAMG rats.

Discussion

The present study confirmed that curcumin can ameliorate clinical symptoms of EAMG rats and protect AchR from being destroyed in the neuromuscular junction (NMJ) of EAMG rats. Furthermore, this study demonstrated that curcumin regulates the balance of Th1/Th2/Th17/Treg in spleens of EAMG rats.

Experimental autoimmune myasthenia gravis (EAMG), a classic animal model for MG, is a B-cell-mediated and T-cell-dependent autoimmune disease of the neuromuscular junction in which the nicotinic acetylcholine receptor (AchR) acts as an autoantigen [1]. In addition, previous studies have suggested that the secretion of inflammatory cytokines and disordered balance of Th subsets (Th1/Th2/Th17/Treg) are involved in the pathogenesis of EAMG [2]. Th1 cells secrete pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and interleukin-2 (IL-2), while Th2 cells secrete anti-inflammatory cytokines, such as IL-4 and IL-10. Moreover, Th17 cells can secrete novel pro-inflammatory cytokine IL-17 [12, 13] while Treg cells are considered to be anti-inflammatory, suppressing certain immune responses. It has been reported that the balance of T helper type 1 (Th1), Th2, Th17, and regulatory T (Treg) subsets of CD4+ helper T-cells is redistributed by upregulating Th1 and Th17 subsets and down-

regulating Th2 and Treg subsets during the development of EAMG in Lewis rats [2].

Numerous studies have suggested that curcumin regulate Th subsets in several autoimmune diseases of the nervous system. In EAE, the inhibition of EAE by curcumin has been associated with an upregulation of IL-10, $CD4^+CD25^+Foxp3^+$Treg cells in the central nervous system (CNS), as well as lymphoid organs, along with a dose-dependent decrease in secretion of IFN-γ, IL-17 in culture. These findings suggest that curcumin differentially regulates CD4+ T helper cell responses in EAE [8]. In EAN, curcumin significantly ameliorated EAN neurological severity and reduced body weight loss of EAN rats. Curcumin suppressed the autoimmune response in EAN by inhibiting Th1 and Th17 polarization, but not through increasing Th2 or Treg cell polarization [9]. However, the immunoregulatory effects of curcumin on MG and EAMG remain unclear. Therefore, this study investigated the therapeutic effects of curcumin in EAN and its underlying mechanisms.

In conclusion, this study demonstrates that curcumin can ameliorate EAMG by regulating the balance of Th1, Th2, Th17, and Treg, to a certain extent, providing a new therapeutic strategy for treatment of MG.

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Disclosure of conflict of interest

None.

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