Topical Application of Mizoribine Suppresses CD4⁺ T-cell–Mediated Pathogenesis in Murine Dry Eye

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Submitted: August 25, 2017
Accepted: October 28, 2017

PURPOSE. We investigate the effect of topical application of mizoribine (MZR) eye drops on CD4⁺ T-cell–mediated immunity and epithelial damage in ocular surface of dry eye disease (DED).

METHODS. Topical application of MZR or vehicle eye drops was performed in mice subjected to desiccating stress (DS). The phenol red cotton test was used to measure tear production, and Oregon-green-dextran (OGD) staining was performed to assess corneal epithelial barrier function. PAS staining was used to quantify conjunctival goblet cells. Immunofluorescent staining and quantitative (q) RT-PCR were used to assess the expression of matrix metalloproteinase (MMP)-9 in corneal epithelium. qRT-PCR and ELISA were used to assess the production of TNF-α and IL-1β in conjunctiva. Apoptosis in ocular surface was assessed by TUNEL and activation of caspase-8. CD4⁺ T-cell–mediated immunity was evaluated by CD4 immunohistochemistry and production of T helper cytokines, including IFN-γ, IL-13, and IL-17A in conjunctiva.

RESULTS. Compared to vehicle control mice, topical MZR-treated mice showed increased tear production, decreased goblet cell loss, and improved corneal barrier function. Topical application of MZR suppressed the expression of MMP-9 in corneal epithelium and apoptosis in ocular surface, while it had no obvious effect on production of TNF-α and IL-1β in conjunctiva. Topical application of MZR decreased CD4⁺ T cells infiltration, with decreased production of IFN-γ and IL-17A, and increased production of IL-13 in conjunctiva.

CONCLUSIONS. Topical application of MZR could alleviate epithelial damage and CD4⁺ T-cell–mediated immunity in ocular surface of DED.

Keywords: mizoribine, dry eye, inflammation, ocular surface

Dry eye disease (DED) is a multifactorial disease of the tears and ocular surface that is accompanied by increased tear osmolarity and inflammation in the ocular surface.¹ DED has become a serious public health problem affecting 4.5% to 7.8% of the population in the United States²,³ and 23.7% to 35.7% of the population in Asia.⁴–⁶ Typical symptoms reported by patients with DED include photophobia, ocular grittiness, burning, stinging, foreign-body sensation, blurred vision, and so forth.⁷–⁹ DED can become progressively troublesome and exert an increasing burden in financial costs and quality of life as the disease progresses.⁸–¹⁰

Regardless of the initiating etiology of DED, a common pathogenic mechanism of DED is tear hyperosmolarity, which can activate a chain of events resulting in a vicious circle of inflammation with consequent ocular surface damage. CD4⁺ T-cell–mediated immunity makes a prominent contribution to the ocular surface epithelial damage in DED.¹¹ In the chronic dry eye, IFN-γ secreted by T helper (Th)1 cells promotes goblet cell loss, cell apoptosis, and expression of cornified envelope precursor proteins in ocular surface.¹² Th17 cytokine IL-17A can result in accelerated loss of apical corneal epithelial cells and corneal barrier disruption via stimulating the production of matrix metalloproteinases (MMP)-3 and 9 in ocular surface.¹³

Immunosuppressive therapy has gained importance as it disrupts the vicious cycle and restores the ocular surface immune homeostasis in DED. Cyclosporine A (CsA) 0.05% eye drops is the only Food and Drug Administration (FDA)-approved immunosuppressant for chronic DED, which can decrease significantly punctate corneal epithelial fluorescein staining, increase conjunctival goblet cell density, and specifically inhibit the T-cell–dependent immune reactions in clinical and experimental dry eye.¹⁴–¹⁸ Nevertheless, use of CsA for ocular application represents a challenge regarding its pharmaceutical formulation. CsA is a highly hydrophobic molecule, practically insoluble in water, that is difficult to be formulated in a total hydrophilic vehicle. The FDA-approved 0.05% CsA eye drops (Restasis, Allergan Inc., Irvine, CA, USA) is a CsA oil-in-water emulsion, and its topical delivery into the eye is tolerated poorly and is associated in some patients with a burning and stinging sensation.¹⁹

Mizoribine (MZR) is a water-soluble immunosuppressant that has been used widely as a primary immunosuppressive agent in clinical renal transplantation, with results comparable or superior to those of conventional drug-like steroids, cyclosporine and azathioprine,²⁰,²¹ by inhibiting the proliferation of T and B cells. Current research demonstrated that MZR also showed remarkable clinical advantages for the treatment of rheumatoid arthritis,²² discoid lupus erythematosus (DLE),²³ IgA nephropathy,²⁴ and Sjögren’s syndrome. In addition, it has been reported that oral administration of MZR can alleviate
effectively the sicca symptoms, including dry mouth and dry eyes, in Sjögren’s syndrome.25,26

Accordingly, we hypothesized that topical application of MZR eye drops would suppress effectively the ocular surface inflammation in dry eye. Thus, we investigated the effect of topical application of MZR eye drops on CD4+ T-cell-mediated immunity and epithelial damage in the ocular surface using a murine dry eye model induced by desiccating stress (DS).

MATERIALS AND METHODS

Mouse Model of Dry Eye

This research protocol was approved by the Experimental Animal Ethics Committee of Xiamen University, and it conformed to the standards of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. DS was created by subjecting female C57BL/6 (B6) mice aged 8 to 12 weeks (Shanghai SLAC Laboratory Animal Center, Shanghai, China) to subcutaneous injection of 0.5 mg/0.2 ml scopolamine hydrobromide (Catalog no. MB5860; Meilubio, Dalian, China) four times daily (8 AM, 11 AM, 2 PM, and 4 PM) and exposure to an air draft and <40% ambient humidity for 5 days. A group of age- and sex-matched mice that did not receive any treatment to induce dry eye served as nonstressed (NS) controls.

Topical Mizoribine Treatment

To evaluate the effect of mizoribine on ocular surface damage of dry eye, topical mizoribine treatment was performed in B6 mice subjected to DS by application of 5 μL mizoribine (0.025%, 0.05%, or 0.1%; Catalog no. M3047; Sigma-Aldrich Corp., Saint Louis, MO, USA) or vehicle (PBS) drops four times daily for 5 days under DS.

Measurement of Tear Production

Tear production (10 eyes/5 mice/group) was measured using phenol red cotton threads (Zone-Quick; Yokota, Tokyo, Japan) at a same time point (8 AM). The thread was placed on the lower conjunctival fornix at approximately one-third of the lower eyelid distance from the lateral canthus for 15 seconds. The length of the wet red thread was measured in millimeters under the microscope.

Corneal Permeability

Corneal epithelial permeability to Oregon-green-dextran (OGD; Catalog no. D7173; 70,000 MW, Anionic, Lysine Fixable; Invitrogen, Eugene, OR, USA) was assessed in each group (10 eyes/5 mice/group) as reported previously.27 Briefly, 0.5 μL 50 mg/ml OGD was instilled onto the ocular surface 1 minute before euthanasia. Corneas then were rinsed with 1 ml saline 5 times and photographed with a high dynamic and resolution digital camera (AZ100; Nikon, Tokyo, Japan) under fluorescence excitation at 470 nm. The mean intensity of corneal OGD staining in digital images was measured by analysis software (NI Elements; version 4.1; Nikon, Melville, NY, USA) with a 3-mm diameter circle placed in the central cornea.

Histology

For immunofluorescent staining, the eyes and adnexa of mice from each group were excised, embedded in optimal cutting temperature compound (OCT compound; Catalog no.4583; Tissue-tec; Sakura Finetek, Torrence, CA, USA), and flash frozen in liquid nitrogen. Sagittal sections (5-μm thick) were cut with a cryostat (CM1850 UV; Leica, Wetzlar, Germany) and placed on glass slides (Catalog no. 188105; CITOGLAS, Jiangsu, China) that were stored at –80°C. For PAS staining, immunohistochemistry and TUNEL labeling, the eyes and adnexa of mice from each group were excised, fixed in 4% formaldehyde for 24 hours, dehydrated in gradient concentrations of ethanol (70%-100%), and infiltrated with paraffin (Catalog no. P3683; Sigma-Aldrich Corp.) at 60°C. Serial sections were cut from the lateral and medial borders of each paraffin block at a thickness of 6 to 8 um and floated on deionized water at 45°C, and single sections were mounted on glass slides. Slides subsequently were dried at 60°C for 1 hour and stored at room temperature.

Measurement of Goblet Cell

Sections cut from paraffin-embedded globs were stained with PAS reagent (Catalog no.395B-IKT; Sigma-Aldrich Corp.). The number of goblet cells in the whole conjunctiva was counted using NIS Elements software.

Immunofluorescent Staining

Cryosections were evaluated for the expression of MMP-9 in corneal epithelium and activated (AC) caspase-8 in conjunctiva. Samples first were fixed in acetone at –20°C, and then blocked with 2% BSA for 60 minutes at room temperature. After that, tissue samples were incubated with polyclonal rabbit anti-MMP-9 antibody (1:500; Catalog no. ab38898; Abcam, Inc., Cambridge, MA, USA), rabbit anti-AC caspase-8 antibody (1 μg/ml; Catalog no. ab25901; Abcam, Inc.) primary antibodies at 4°C overnight. Negative controls were performed at the same time incubated with PBS in place of primary antibody. The next day, after three washes in PBS (PH 7.2), samples were incubated with appropriate Alexa-Fluor 594 anti-goat IgG (1:300; Catalog no. A11055; Invitrogen) or anti-rabbit IgG (1:500; Catalog no. A21200; Invitrogen) for 1 hour in the dark at room temperature. Samples then were counterstained with 4’6-diamidino-2-phenylendole (DAPI; Catalog no. H-1200; Vector, Burlingame, CA, USA) for 5 minutes. Digital images of representative areas of the central cornea and conjunctival fornix were captured with a Leica upright microscope (DM2500; Leica Microsystems), and fluorescent intensity was analyzed using the NIS Elements Software.

Immunohistochemistry

Immunohistochemistry was performed to detect and count the CD4-positive cells in conjunctiva. Cryosections were blocked with 20% goat serum for 30 minutes, stained with purified rat anti-mouse CD4 (1:50; Catalog no. 553647; BD Pharmingen, San Diego, CA, USA) primary antibody for 60 minutes, biotinylated secondary antibodies goat anti-rat antibody (1:25; Catalog no. 559286; BD Pharmingen) for 30 minutes, and Vectastain Elite ABC using NovaRed reagents (Catalog no. PK-6100; Vector, Burlingame, CA) according to the instructions. Secondary antibody alone and appropriate anti-mouse isotype (BD Pharmingen) controls also were performed.

Three representative pictures of each animal were examined and photographed with a microscope equipped with a digital camera (Eclipse E400 with a DS-Fi1; Nikon, Melville, NY, USA). Positively stained cells were counted in conjunctiva using NIS Elements Software. The results were expressed as the total number of positively stained cells in conjunctiva.

TUNEL Assay

TUNEL assay was performed using a commercially available kit (DeadEnd Fluorometric TUNEL System; Catalog no. G3250;
Promega, Madison, WI, USA). Digital images of representative areas were captured with a Leica upright microscope (DM2500; Leica Microsystems), and were analyzed using the NIS Elements Software. The results were expressed as the number of positively stained cells in corneal epithelium and conjunctiva.

Total RNA Extraction, Reverse Transcription and Quantitative Real-Time PCR
Total RNA collected from the corneal epithelium and conjunctiva was extracted using PicoPure RNA isolation kit (Catalog no. KIT0204; Arcturus, USA) according to the manufacturer’s instructions. Five samples per group/stain were used, and one sample consisted of pooled samples from both eyes of one mouse. The RNA concentration was measured based on its optical density at 260 nm and stored at −80°C before use. First-strand cDNA was synthesized using a reverse transcription kit (Catalog no. RR047A; TaKaRa, Shiga, Japan).

Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Alameda, CA, USA), and the parameters consisted of predenaturation at 95°C for 60 seconds, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing, and extension at 60°C for 30 seconds. After that, a melt curve analysis was conducted to check amplification specificity. The primers are provided in the Table. Samples and standards were assessed in duplicate. The results were analyzed using the comparative threshold cycle (CT) method and normalized with β-actin as an endogenous reference.

ELISA Test
The conjunctival proteins were extracted with cold RIPA buffer (Catalog no. R0278; Sigma-Aldrich Corp.), and total protein concentration was measured with a BCA protein assay kit (Catalog no. 23225; Thermo Fisher Scientific, Waltham, MA, USA). The same dilution ratio was used in all groups. Each group contained five samples, and each sample consisted of pooled conjunctiva of both eyes of the same animal. ELISA kits were used to detect the protein concentrations of TNF-α (Catalog no. BMS607-3; eBioscience, San Diego, CA, USA), IL-1β (Catalog no. BMS6002; eBioscience), IL-17A (Catalog no. BMS6015; eBioscience), and IFN-γ (Catalog no. BMS606; eBioscience) according to the manufacturer’s instructions. The assay was performed in duplicates to ensure data reproducibility.

Western Blot
The conjunctival proteins were extracted and measured as described above. Each sample consisted of pooled conjunctiva of both eyes of two animals. Equal amounts of proteins were subjected to electrophoresis and then transferred electronical-ly to polyvinylidine fluoride (PVDF) membranes (Catalog no. IPVH00010; Millipore, Billerica, MA, USA). After 1 hour of blocking in 2% BSA, the membranes were incubated overnight at 4°C with anti-Caspase-8 primary antibody (Catalog no. ab25901; Abcam Inc.) and β-actin (1:10,000; Sigma-Aldrich Corp.) as a loading control. Next day, after three washes with Tris-buffered saline with 0.05% Tween 20 for 10 minutes each, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000; Catalog no. a0545; Sigma-Aldrich Corp.) for 1 hour at room temperature. The specific binds were visualized by enhanced chemiluminescence reagents (Catalog no. ECL-500; ECL, Lulong, Inc., Xiamen, China) and the image intensity was calculated by the transilluminator (Chemidoc XRS System; Bio-Rad Laboratories, Philadelphia, PA, USA).

Statistical Analysis
Data are represented as mean ± SD. Statistical significance was evaluated by 1-way ANOVA with Tukey’s post hoc test using GraphPad Prism 5.0 software (GraphPad Software; San Diego, CA, USA). P ≤ 0.05 was considered statistically significant.

RESULTS

The Effects of Topical Application of Mizoribine on Tear Production During DS
The phenol red thread test was used to assess the effect of mizoribine eye drops on tear production in DS-induced murine dry eye in our study. Compared to the NS group, the DS group showed significantly decreased tear production (Fig. 1A; DS vs. NS, 4.1 ± 1.3 mm vs. 1.0 ± 0.7 mm; P < 0.01). Topical application of 0.05% mizoribine significantly increased the tear production in B6 mice under DS (Fig. 1A; DS+PBS vs. DS+0.05% mizoribine, 1.2 ± 0.7 mm vs. 2.1 ± 0.6 mm; P < 0.05). Based on the results of tear production, 0.05% was treated as the adopted concentration to assess the effect of mizoribine on ocular surface damage in dry eye induced by DS.

The Effects of Topical Application of Mizoribine on Conjunctival Goblet Cell Loss During DS
Compared to the NS group, the DS group showed significantly decreased numbers of conjunctival goblet cells (Figs. 1B, 1C; NS vs. DS, 100 ± 14 vs. 45 ± 19; P < 0.01). Topical application of 0.05% mizoribine significantly increased the number of conjunctival goblet cells under DS (Figs. 1B, 1C; DS+vehicle vs. DS+0.05% mizoribine, 45 ± 6 vs. 68 ± 12; P < 0.05).

The Effects of Topical Application of Mizoribine on Corneal Barrier Function During DS
As shown in Figures 2A and 2B, the mean intensity of corneal OGD staining significantly increased in the DS group (6.32 ±
FIGURE 1. The effects of topical application of mizoribine on tear production and goblet cell loss during DS. Tear production was measured by phenol red thread test (A). Mean goblet cell numbers (B) and representative images of PAS staining (C) in conjunctiva. Data were shown as mean ± SD. *P < 0.05, **P < 0.01.

FIGURE 2. The effects of topical application of mizoribine on corneal barrier function during DS. Representative images (A) and mean intensity (B) of OGD staining. The level of MMP-9 mRNA (C) of corneal epithelium. Mean intensity (D) and representative images (E) of MMP-9 immunofluorescence staining. Data were shown as mean ± SD. *P < 0.05, **P < 0.01.
1.37) compared to the NS group (2.97 ± 0.47, P < 0.01). Topical application of 0.05% mizoribine significantly decreased the intensity of corneal OGD staining (Figs. 2A, 2B; DS+vehicle vs. DS+0.05% mizoribine, 6.94 ± 1.04 vs. 4.17 ± 0.76; P < 0.01). As reported previously, MMP-9 is the key factor in corneal barrier disruption in DS-induced DED. This study investigated the expression of MMP-9 in corneal epithelium using immunofluorescent staining and quantitative (q) RT-PCR, and found that topical application of 0.05% mizoribine suppressed the expression of MMP-9 in corneal epithelium during DS (Figs. 2C–E). These results, taken together, suggested that topical application of 0.05% mizoribine could improve corneal barrier function during DS.

**The Effects of Topical Application of Mizoribine on Production of TNF-α and IL-1β in Conjunctiva During DS**

The production of TNF-α and IL-1β in conjunctiva was increased significantly in the DS compared to the NS groups (Fig. 3; P < 0.05 for each comparison). No significant difference was noted in the production of TNF-α or IL-1β in the conjunctiva between mice treated with 0.05% mizoribine and vehicle eye drops (Fig. 3; P > 0.05 for each comparison). Interestingly, ELISA showed significantly decreased production of TNF-α and IL-1β in vehicle eye drop-treated mice compared to DS mice (Fig. 3; P < 0.01 for each comparison).

**The Effects of Topical Application of Mizoribine on CD4⁺ T-cell–mediated Inflammation in Conjunctiva During DS**

DED is an inflammatory ocular surface disease characterized by infiltration of CD4⁺ T cells producing IFN-γ and IL-17A in conjunctiva. Our results indicated that topical application of 0.05% mizoribine effectively suppressed the DS-induced infiltration of CD4⁺ T cells in conjunctiva (Figs. 4A, 4B). The qRT-PCR and ELISA assay showed that topical application of 0.05% mizoribine decreased production of IL-17A and IFN-γ and increased production of IL-13 in conjunctiva under DS (Fig. 4C–H).

**The Effects of Topical Application of Mizoribine on Apoptosis in Ocular Surface During DS**

Topical application of 0.05% mizoribine effectively decreased immunoreactivity and protein levels of AC-caspase-8 in conjunctiva (Figs. 5A–D). The TUNEL assay showed that the 0.05% mizoribine–treated mice had less apoptotic cells than the PBS-treated mice in corneal and conjunctival epithelium (Figs. 5E–H). These evidences, taken together, suggested that mizoribine can suppress DS-induced apoptosis in the ocular surface.

**DISCUSSION**

MZR is a water-soluble imidazole nucleoside with immunosuppressive activity. Our findings demonstrated that topical...
application of MZR could alleviate ocular surface damage effectively using a murine DED model induced by DS. Topical application of MZR decreased conjunctival CD4\(^+\) T-cell infiltration, increased tear production, decreased goblet cell loss, suppressed ocular surface cell apoptosis, and improved corneal barrier function during DS.

The pathogenesis of DED is a multifactorial process that includes activation of various inflammatory pathways in the ocular surface epithelia by the hyperosmolar tear film and cytokines produced by resident intraepithelial lymphocytes and infiltrating Th1 and Th17 cells. In the DED model used in our study, it has been demonstrated previously that DS-activated CD4\(^+\) T cells that, when adoptively transferred to naive T-cell–deficient nude mice, were sufficient to elicit autoimmune lacrimal keratoconjunctivitis sicca with features resembling clinical DED,\(^29\) indicating that CD4\(^+\) T cells make a prominent contribution to mucosal inflammation and ocular surface damage in DED. MZR is an imidazole nucleoside and an immunosuppressive agent. Because of its relative lack of toxicity, during the past decade MZR has been used
frequently as a component of immunosuppressive drug regimens. The immunosuppressive effect of MZR has been suggested to be due to inhibition of DNA synthesis in the S phase of the cell cycle in T and B cells by affecting the synthesis of inosine monophosphate (IMP) dehydrogenase and guanosine monophosphate (GMP) synthetase, both enzymes being required for synthesis of GMP from IMP in the de novo pathway.30 Sakaguchi et al. 31 investigated the growth inhibitory effect of MZR on several cell lines and showed that MZR had a strong inhibitory effect on lymphoma cell line L5178Y and L-cells, with IC50 values >100. Our study found that topical application of MZR decreased conjunctival CD4+ T-cell infiltration, suggesting topical application of MZR can suppress the generation of CD4+ T cells in ocular surface during DS.

Th1 cells secreting IFN-γ were the first pathogenic lymphocyte subset identified in experimental and clinical DED. Previously, it has been confirmed that IFN-γ has a pivotal

**FIGURE 5.** The effects of topical application of mizoribine on apoptosis in ocular surface during DS. Representative images (A), mean fluorescence intensity (B), and protein level (C, D) of AC-caspase-8 in conjunctiva. Representative images of TUNEL staining (E, G) and the number of TUNEL-positive cells (F, H) in cornea and conjunctiva. Data were shown as mean ± SD. *P < 0.05, **P < 0.01.
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role in exacerbating conjunctival apoptosis in DED.32 Evidence includes: (1) DS induces apoptosis in the conjunctival epithelia that expresses IFN-γ through a caspase-8-mediated intrinsic apoptotic pathway; (2) IFN-γ knockout mice showed resistance to DS-induced apoptosis in conjunctiva; and (3) Exogenous administration of IFN-γ increased DS-induced apoptosis in the conjunctiva in wild type and IFN-γ knockout mice. Our study showed that topical application of MZR suppressed caspase-8-mediated apoptosis and the production of Th1 cytokine IFN-γ in ocular surface. Accordingly, we speculated that topical application of MZR suppressed cell apoptosis mainly via decreased production of Th1 cytokine IFN-γ in the ocular surface during DS.

Th17 cells also appear to be key drivers of chronic DED. In DED mice, Th17 cells are present in the draining cervical lymphatic nodes and within the ocular surface. It has been shown that IL-17A contributes to development of corneal epithelium via increased MMP-9 production and activation in the DS model used in our study.13 MMP-9 has been found to have a central role in DS-induced corneal barrier disruption, as MMP-9-deficient mice were resistant to barrier disruption in DS.33,34 IL-17 neutralization dampened MMP-9 expression and blocked breakdown of corneal epithelial tight junctions during DS.13 In our study, topical application of MZR suppressed the production of IL-17A and MMP-9 in the ocular surface, which may contribute to improvement of corneal barrier function in mice following MZR eye drops treatment during DS.

Consistent with a previous report,26 DS promoted the production of TNF-α and IL-1β in conjunctiva. Topical application of MZR had no obvious effect on the production of TNF-α and IL-1β in conjunctiva during DS in our study. Interestingly, our study showed that topical vehicle eye drops treatment decreased production of TNF-α and IL-1β in conjunctiva during DS. We speculated that isotonic aqueous solution may alleviate tear hyperosmolarity and, accordingly, suppress inflammatory cytokine production in the ocular surface during DS.

Goblet cells are simple columnar secretory epithelial cells that secrete gel-forming mucins to form the mucous component of the tear film. Although the cause of goblet cell loss in DED has not been fully established, evidence suggests that it may be due to the decreased ratio of Th2/Th1 cytokine (IL-13/IFN-γ).35,36 IL-13 has been demonstrated to have a homeostatic role in promoting goblet cell differentiation.37 By contrast, IFN-γ can result in goblet cell loss via stimulating apoptosis and suppressing IL-13 signaling. Our findings showed that topical MZR treatment increased production of IL-13 and decreased production of IFN-γ, which may certainly rescue the goblet cell loss during DS.

In summary, our findings supported the potential clinical application of MZR eye drops for treatment of DED, and provided new insight for clinical immunosuppressive therapy in ocular surface disease. Further investigations are needed to compare the therapeutic effect of 0.05% MZR eye drops to commercially available 0.05% cyclosporine A eye drops in DED.

Acknowledgments

Supported by grants from the National Key Scientific Research Project (No.201302015, ZL) and National Natural Science Foundation of China (No. 81500093, XZ and No. 81330022, ZL).

Disclosure: X. Zhang, None; X. Lin, None; Z. L. Liu, None; Y. Wu, None; Y. Yang, None; W. Ouyang, None; W. Li, None; Z. Liu, None

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