Cornea

Rapamycin Rescues Endoplasmic Reticulum Stress–Induced Dry Eye Syndrome in Mice

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PURPOSE. To investigate whether rapamycin protects tear production and the ocular surface during endoplasmic reticulum (ER) stress–induced dry eye syndrome in mice.

METHODS. Tunicamycin was injected intraperitoneally in BALB/c mice without or with rapamycin (TM or RM5 group). Peritoneal injection of PBS performed in vehicle group. Group without injection served as control. Blinking rate, fluorescein staining score (FSS), and phenol red thread tear production test were measured at 4 days, 1 week, and 2 weeks after treatment. Levels of inflammatory and angiogenic cytokines were measured by ELISA.

RESULTS. Blinking rate and FSS were elevated, and tear production was decreased in TM group compared with controls (P < 0.05 for all), which was ameliorated by rapamycin at 1 and 2 weeks. Levels of inflammatory and angiogenic cytokines in the cornea and lacrimal glands were higher in the TM group than controls, and lower in the RM5 group than the TM group at 1 and 2 weeks (P < 0.05 for all).

CONCLUSION. Rapamycin protected tear production and the ocular surface against this dry eye syndrome by ameliorating ER stress–induced vascular damage and inflammation of lacrimal glands and the ocular surface.

Keywords: rapamycin, dry eye syndrome, ER stress, inflammation, lacrimal glands

D y dry eye syndrome is a disorder of the tear film and ocular surface, accompanied by ocular discomfort, tear instability, and ocular surface inflammation.1 The pathophysiology of dry eye syndrome includes oxidative stress and inflammation of the lacrimal glands, ocular surface, and eyelid.2,3 Dry eye syndrome is associated with aging, depression, irritable bowel syndrome, obesity, menopause, and vitamin D deficiency.4 Recently, endoplasmic reticulum (ER) stress has been suggested as a pathophysiology of dry eye syndrome.5,6 ER stress has been described in many diseases, including cardiovascular disorders, neurodegenerative diseases, inflammatory bowel disease, and rheumatoid arthritis.7,8 The ER is a cellular organelle in which proteins and lipids are synthesized and processed, and metabolism of xenobiotic compounds occurs.8–10 Newly synthesized proteins are folded in the ER into the three-dimensional configuration and trafficked according to their destination in the cell.11,12 Failures in this quality control result in an increase of unfolded proteins in the ER, which is known as ER stress.11,12 Prolonged or severe ER stress causes apoptosis and promotes the progression of disease by affecting the function and structure of proteins and lipids.11 ER stress induction as well as inflammation in lacrimal glands is important in the pathogenesis of dry eye syndrome or Sjögren’s syndrome.13,14 ER stress also may be linked to inflammation of ocular surface and lacrimal glands, which is a major mechanism of dry eye. ER stress in salivary glands plays an essential role in Sjögren syndrome.15 Induction of ER stress is useful for a dry eye model. Nevertheless, there has been no study reporting ER stress–induced dry eye model. In this study, we reported the ER stress–induced dry eye model using tunicamycin.

Lacrimal glands produce and secrete lacrimal fluid that constitutes the tears.15 The aqueous layer of tears over the ocular surface comprises 90% of the tear volume.15 A decrease of lacrimal fluid secretion leads to tear deficiency and ocular surface damage, which is known as dry eye syndrome.16 Lacrimal glands are composed of 80% acinar cells, 12% ductal cells, and 8% nerves and vessels.15 Nerves and blood vessels surround the basolateral side of the acinar cells and initiate secretion.17 Lacrimal glands are innervated by predominantly parasympathetic nerves, and they are highly vascularized.16 Thus, aging and vascular events may lead to impairment of the functions of lacrimal glands.17

Rapamycin is an immunosuppressant used to prevent the rejection in organ transplantation.18 Rapamycin reduces inflammation and induces autophagy by inhibiting mammalian target of rapamycin (mTOR).19,20 mTOR modulates the cell functions such as cell proliferation, lipid synthesis, protein synthesis, and cell death. mTOR mediates inflammation through regulating inflammatory cytokines such as IL-6 and IFN-γ. Furthermore, rapamycin protects against vascular occlusion and ischemic injury by modulating apoptosis and autophagy.19 Therefore, rapamycin can be expected to be suitable for preventing dry eye syndrome induced by ER stress. In this study, we investigated whether rapamycin protects tear...
production and the ocular surface against ER stress-induced dry eye syndrome in mice.

MATERIALS AND METHODS

Animals

This study was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old female BALB/c mice were obtained from Samtako Bio Korea (Seoul, Korea). Mice were maintained in a colony room with 12/12-hour light/dark cycles at 25°C for 7 days before initiating experiments.

Induction of ER Stress, Drug Preparation, and Treatment Protocol

Tunicamycin (1 mg/kg; 0.1 mL intraperitoneal injection of 0.2 mg/mL) was injected intraperitoneally twice a week in the TM group. Mice in the RM5 group received a peritoneal injection of rapamycin (5 mg/kg; 0.1 mL intraperitoneal injection of 1 mg/mL) and tunicamycin (1 mg/kg; 0.1 mL intraperitoneal injection of 0.2 mg/mL) twice a week. Tunicamycin-induced ER stress has been reported to decrease saliva secretion in mice and to be able to serve as dry eye syndrome. Twenty eyes of 10 mice were used for each group. Intraperitoneal injection of 0.1 mL PBS was performed in the vehicle group. The group that did not undergo any injection served as control (Sham). Clinical evaluations were performed for 4 days, 1, 2, and 2 weeks, and then mice were killed for molecular and histological evaluation.

Clinical Evaluation

Blink rate was observed over a 60-second interval at 25°C under 55% to 65% humidity. The number of blinks was measured three times and averaged. The tear production test was performed using phenolsulfonphthalein-saturated cotton threads (PRT test; Nourishi Optical, Danyang City, China). PRTs were applied in the lateral canthus of eye, using forceps, for 30 seconds in an unanesthetized mouse. The length of wetted thread was then measured in millimeters using digital calipers.21 Corneal unanesthetized mouse. The length of wetted thread was then measured using digital calipers.21 Corneal excised and fixed in 3.7% formaldehyde in PBS. Hematoxylin and eosin staining was performed, and the slides were observed under an optical microscope (Olympus, Tokyo, Japan).

Immunohistochemical staining for IL-6, IFN-γ, X-box binding protein 1 (XBP-1), 78-kDa glucose regulated protein (GRP78), hypoxia-inducible factor (HIF-1α), CD4, and CD11b was performed. For immunohistochemistry, the tissue sections were hydrated and antigen retrieval was performed using microwave oven in citrate buffer (pH 6.0). Tissue sections were blocked with normal horse serum for 1 hour at room temperature and then, primary antibody for IL-6 (ab60672, Abcam, Cambridge, MA, USA), IFN-γ (sc-375727; Santa Cruz Biotehnologies, Santa Cruz, CA, USA), XBP-1 (sc-7160; Santa Cruz), GRP78 (MABC675; Merck Millipore, Billerica, MA, USA), HIF-1α (sc-9989; Santa Cruz), CD4 (sc-19641; Santa Cruz), and CD11b (ab8878; Abcam) was applied at 4°C overnight. Secondary biotinylated antibody was applied for 2 hours at room temperature. Vectorstain Universal Elite ABC staining kit (Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidine peroxidase substrate (Vector Laboratories) was used for immunodetection. Hematoxylin staining was performed as nuclear counterstaining.

Enzyme-Linked Immunosorbent Assay

Samples were sonicated in radioimmunoprecipitation assay (RIPA) buffer (Biosesang, Seoul, Korea), containing phosphate inhibitor cocktail and protease inhibitor cocktail. Specimens were centrifuged at 14,000g at 4°C for 20 minutes and stored at −70°C. ELISA kits for IL-6, IFN-γ, HIF-1α, and VEGF were bought from R&D Systems (Minneapolis, MN, USA). A double-sandwich ELISA was done according to manufacturer’s protocol. Cytokine concentrations were normalized to total protein concentrations. A biocinchonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) was used for total protein quantification.

Western Blot

Samples were sonicated in RIPA buffer (Bioesang, Seoul, Korea), containing phosphate inhibitor cocktail and protease inhibitor cocktail. Specimens were centrifuged at 14,000g at 4°C for 20 minutes and stored at −70°C. The protein concentration was measured using the Pierce bicinchonic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Western blotting for the measurement of activating transcription factor 6 (ATF6), AKT, platelet and endothelial cell adhesion molecule 1 (PECAM-1), protein kinase RNA-like ER kinase (PERK), phosphorylated PERK (p-PERK), XBP-1, CCAAT-enhancer-binding protein homologous protein (CHOP), GRP78, vascular endothelial [VE]-cadherin, microtubule-associated protein 1A/1B-light chain 3 (LC3), β-actin, and GAPDH levels was performed using standard methods. Five percent skim milk was used for blocking the nonspecific binding at 25°C for 1 hour. After washing, the membrane was incubated with primary antibody at 4°C overnight. ATF6 (PA5–20215; Thermo Fisher), AKT (4691; Cell Signaling, Danvers, MA, USA), PECAM-1 (sc-8306; Santa Cruz), PERK (sc-13073, Santa Cruz), p-PERK (sc-52777, Santa Cruz), XBP-1 (sc-7160, Santa Cruz), CHOP (MA1–250; Thermo Fisher, Waltham, MA, USA), GRP78 (MABC675), VE-cadherin (sc-9989; Santa Cruz), LC3 (M186–3; MBL International, Woburn, MA, USA), β-actin (ab8227; Abcam), and GAPDH (LP-PA0212; AbFrontier, Seoul, Korea) primary antibodies were all raised in rabbit. After washing, alkaline

Hematoxylin and Eosin Staining and Immunohistochemistry

Extracted lacrimal glands and ocular surface tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections of 5 μm thick were used for histological stainings. Histological staining was performed using four eyes from each group. Briefly, the cornea and lacrimal glands were excised and fixed in 3.7% formaldehyde in PBS. Hematoxylin and eosin staining was performed, and the slides were observed under an optical microscope (Olympus, Tokyo, Japan).
phosphatase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (Bio-Rad Laboratories, Hercules, CA, USA) was used as secondary antibody; 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Promega, Madison, WI) were used for detection of bands. Experiments were repeated three times.

Statistics

All data are expressed as mean ± SD. The SPSS version 23.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analyses. Independent t-tests were used to compare measurements between groups. The P value for statistical significance was defined as P < 0.05.

Results

Rapamycin Reduced Inflammation of the Ocular Surface

Blinking rate was increased in the TM group (8.6 ± 2.4) more than the sham (0.8 ± 1.3) or the vehicle group (0.8 ± 0.8) or the RM5 group (4.4 ± 2.1) at 4 days (P < 0.001, < 0.001, and 0.015, respectively, independent t-tests; Fig. 1A). Blinking rate was 97.5% higher in the TM group than the sham. Blinking rate was also 450% higher in the RM5 group compared with the vehicle group (P = 0.018). At 1 week, blinking rate was increased in the TM group (8.1 ± 1.7) than the sham (1.2 ± 0.8) or the vehicle group (3.4 ± 0.9) or the RM5 group (4.3 ± 1.4) (P < 0.001 for all comparisons). Blinking rate was 57.5% higher in the TM group than the RM5 group. At 2 weeks, blinking rate was increased in the TM group (7.5 ± 4.7) than the sham (1.0 ± 1.3) or the vehicle group (1.5 ± 1.4) or the RM5 group (2.0 ± 2.1) (P < 0.001, 0.019 and 0.034, respectively). Blinking rate was 650% higher in the TM group than the RM5 group. Corneal fluorescein staining is shown in Figure 1B. FSS was higher in the TM group (1.3 ± 0.5) compared with the sham (0.0 ± 0.0) or the vehicle group (0.0 ± 0.0) at 4 days (P = 0.002 for both). At 1 week, FSS was higher in the TM group (3.0 ± 0.0) and in the RM5 group (1.6 ± 0.5) compared with the sham (0.0 ± 0.0) or the vehicle group (0.0 ± 0.0) (P < 0.001, < 0.001, and 0.005, respectively). However, FSS was 47% lower in the RM5 group than the TM group at 1 week (P = 0.005). At 2 weeks, FSS was higher in the TM group (2.6 ± 0.5) compared with the sham (0.2 ± 0.4) or the vehicle group (0.2 ± 0.4) or the RM5 group (0.9 ± 0.9) (P < 0.001 for all comparisons). FSS was 170% higher in the TM group than the RM5 group.

The levels of inflammatory cytokines of the ocular surface were measured by ELISA (Fig. 2). IL-6 levels were higher in the TM group (0.031 ± 0.002 pg/µg total protein) compared with the sham (0.015 ± 0.005 pg/µg total protein) or the vehicle group (0.021 ± 0.004 pg/µg total protein) at 4 days (P < 0.001 and 0.006; Fig. 2A). At 1 week, IL-6 levels of the ocular surface were higher in the TM group (0.045 ± 0.014 pg/µg total protein) compared with the sham (0.020 ± 0.010 pg/µg total protein) or the vehicle group (0.020 ± 0.005 pg/µg total protein) (P = 0.027 and 0.035, respectively), but were 36% lower in the RM5 group (0.029 ± 0.007 pg/µg total protein) compared with the TM group (P = 0.027). At 2 weeks, IL-6 levels were higher in the TM group (0.029 ± 0.008 pg/µg total protein) compared with the sham (0.018 ± 0.004 pg/µg total protein) or the vehicle group (0.015 ± 0.005 pg/µg total protein) (P = 0.025 and 0.006, respectively), but were 41% lower in the RM5 (0.017 ± 0.006 pg/µg total protein) compared with the TM group (P = 0.019). INF-γ levels of the ocular surface were higher in the TM group (26.68 ± 4.14 pg/µg total protein) compared with the sham (8.75 ± 6.92 pg/µg total protein) or the vehicle group (9.76 ± 9.42 pg/µg total protein) at 4 days (P = 0.001 and 0.031, respectively). At 1 week, INF-γ levels were higher in the TM group (27.69 ± 7.99 pg/µg total protein) compared with the sham (9.11 ± 5.37 pg/µg total protein) or the vehicle group (11.54 ± 7.94 pg/µg total protein) (P = 0.003 and 0.045, respectively), but were 48% lower in the RM5 group (14.49 ± 7.81 pg/µg total protein) than the TM group (P = 0.018). At 2 weeks, INF-γ levels were higher in the TM group (32.55 ± 8.07 pg/µg total protein) compared with the sham (9.91 ± 3.14 pg/µg total protein) or the vehicle group (10.67 ± 7.96 pg/µg total protein) (P < 0.001 and 0.006, respectively), but were 42% lower in the RM5 (16.16 ± 9.62 pg/µg total protein) than the TM group (P = 0.019).

Immunohistochemistry revealed that XBP-1 and GRP78 expression in cornea and in conjunctiva was increased in the TM group compared with RM5 group (Fig. 2C). IFN-γ
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Tear production measured by the phenol red test decreased in the TM group (1.27 ± 0.28 mm) and in the RM5 group (1.62 ± 0.25 mm) compared with the sham (2.57 ± 0.52 mm) or the vehicle group (2.49 ± 0.15 mm) at 4 days (P < 0.001 for all comparisons; Fig. 3A); however, tear production was not different between the TM and RM5 groups at 4 days. At 1 week, tear production was reduced in the TM group and RM5 group compared with the sham (0.73 ± 0.12 mm in RM5 group, and 2.67 ± 0.37 mm in the sham or the vehicle group, P < 0.001 for all). At 2 weeks, tear production was reduced in the TM group (1.32 ± 0.24 mm) compared with the sham (2.30 ± 0.43 mm) or the vehicle group (2.68 ± 0.53 mm) (P = 0.002 and < 0.001, respectively), and elevated by 73% in the RM5 group (2.28 ± 0.58 mm) compared with the TM group (P = 0.024). The size of lacrimal glands was smaller in the TM group (0.62 ± 0.08 mm) and RM5 groups (0.74 ± 0.10 mm) than the sham (0.98 ± 0.06 mm) or the vehicle group (1.00 ± 0.13 mm) at 4 days (P < 0.001 for both comparisons; Fig. 3B). The lacrimal glands were 19% larger in the RM5 group compared with the TM group at 4 days (P = 0.020). At 1 week, the size of lacrimal glands was smaller in the TM group (0.48 ± 0.06 mm) and in the RM5 group (0.60 ± 0.07 mm) compared with the sham (0.94 ± 0.08 mm) or the vehicle group (1.00 ± 0.12 mm) (P < 0.001 for both comparisons). The lacrimal glands were 25% larger in the RM5 than the TM group at 1 week (P = 0.011). At 2 weeks, the size of lacrimal glands was smaller in the TM group (0.65 ± 0.06 mm) and RM5 group (0.78 ± 0.09 mm) the sham (0.99 ± 0.04 mm) or the vehicle group (1.00 ± 0.08 mm) (P < 0.001 for all comparisons). The lacrimal glands were 24% larger in the RM5 group than the TM group at 2 weeks (P = 0.001). p-PERK expression was elevated in the TM and RM5 groups at 4 days and then reduced only in the RM5 group at 1 week (Fig. 3C). p-PERK expression was 49% and 63% lower in RM5 group (190.61% ± 58.16% at 1 week and 161.03% ± 10.07% at 2 weeks) compared with the TM group (373.90% ± 42.74% at 1 week and 430.38% ± 86.84% at 2 weeks) (P = 0.012 and 0.006, respectively, independent t-test).

PECAM-1 expression was lower in the TM groups (19.54% ± 0.98%) compared with the sham and 341% higher in RM5 group (190.61% ± 58.16% at 1 week and 161.03% ± 10.07% at 2 weeks) compared with the TM group (373.90% ± 42.74% at 1 week and 430.38% ± 86.84% at 2 weeks) (P = 0.012 and 0.006, respectively, independent t-test).
independent \( t \)-test; Fig. 4B). LC3-II/LC3-I ratio was increased by 198\% in the RM5 group (522.71 ± 183.29\%) compared with the TM group (175.53 ± 92.04\%, \( P = 0.043 \), independent \( t \)-test; Fig. 4C). At 4 days, HIF-1\( \alpha \) levels of lacrimal glands were higher in the TM group (0.31 ± 0.04 pg/\(\mu\)g total protein) compared with the sham (0.26 ± 0.01 pg/\(\mu\)g total protein) or the vehicle group (0.25 ± 0.04 pg/\(\mu\)g total protein) (\( P = 0.014 \) or 0.035, respectively; Figs. 4D, E), but were not different between the TM and RM5 groups. At 1 week, HIF-1\( \alpha \) levels of lacrimal glands were higher in the TM group (0.35 ± 0.03 pg/\(\mu\)g total protein) compared with the sham (0.21 ± 0.07 pg/\(\mu\)g total protein) or the vehicle group (0.21 ± 0.07 pg/\(\mu\)g total protein; \( P < 0.001 \) and \( P = 0.001 \), respectively), and were 17\% lower in the RM5 group (0.29 ± 0.05 pg/\(\mu\)g total protein) compared with the TM group (\( P = 0.049 \)). At 2 weeks, HIF-1\( \alpha \) levels of lacrimal glands were higher in the TM group (0.26 ± 0.02 pg/\(\mu\)g total protein) compared with the sham (0.20 ± 0.02 pg/\(\mu\)g total protein) or the vehicle group (0.23 ± 0.01 pg/\(\mu\)g total protein) (\( P = 0.006 \) and 0.019, respectively), and were 23\% lower in the RM5 group (0.20 ± 0.03 pg/\(\mu\)g total protein).
compared with the TM ($P = 0.020$). At 4 days, VEGF levels of lacrimal glands were higher in the TM group (3.96 ± 0.51 pg/μg total protein) compared with the sham (2.41 ± 0.39 pg/μg total protein) or the vehicle group (2.88 ± 0.54 pg/μg total protein) ($P < 0.001$ and $0.022$, respectively), and were 12% lower in the RM5 group (3.29 ± 0.27 pg/μg total protein) compared with the TM group ($P = 0.020$). At 2 weeks, VEGF levels of lacrimal glands were higher in the TM group (3.40 ± 0.34 pg/μg total protein) compared with the sham (2.17 ± 0.39 pg/μg total protein) or the vehicle group (2.76 ± 0.47 pg/μg total protein) ($P < 0.001$ and 0.048, respectively), and were 19% lower in the RM5 group (2.74 ± 0.22 pg/μg total protein) compared with
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**Rapamycin Prevents ER Stress–Induced Inflammation of Lacrimal Glands**

Hematoxylin and cosin staining showed that acinar atrophy, indicated by shrunken acinar cells with no secretory granules, and ductal infiltration of inflammatory cells, such as neutrophils and macrophages, was present in the TM group (Fig. 5A). Intact acinar cells and fewer infiltrating inflammatory cells were observed in the RM5 group compared with the TM group. CD11b+ cells and CD4+ cells infiltrated into lacrimal glands in the TM group (Figs. 5B, C). GRP78 of lacrimal gland increased in TM group (Fig. 5D).

At 4 days, IL-6 levels of lacrimal glands were elevated in the TM group (0.082 ± 0.022 pg/μg total protein) compared with the sham (0.050 ± 0.005 pg/μg total protein) or the vehicle group (0.055 ± 0.008 pg/μg total protein) (P = 0.006 and 0.047, independent t-test; Fig. 5E), but were not significantly different between the TM and RM5 groups. At 1 week, IL-6 levels of lacrimal glands were elevated in the TM group (0.088 ± 0.035 pg/μg total protein) compared with the sham (0.050 ± 0.012 pg/μg total protein) or the vehicle group (0.049 ± 0.010 pg/μg total protein) (P = 0.030 and 0.010, respectively), and were reduced by 43% in the RM5 group (0.050 ± 0.015 pg/μg total protein) compared with the TM group (P = 0.041). IL-6 levels were not different between the sham or the vehicle group and RM5 groups at 1 week. At 2 weeks, IL-6 levels of lacrimal glands were higher in the TM group (0.105 ± 0.028 pg/μg total protein) compared with the sham (0.049 ± 0.011 pg/μg total protein) or the vehicle group (0.067 ± 0.009 pg/μg total protein) (P = 0.001 and 0.007, respectively), and were reduced by 45% in the RM5 group (0.058 ± 0.034 pg/μg total protein) compared with the TM group (P = 0.051). IL-6 levels were not different between the sham or the vehicle group and RM5 groups at 2 weeks. At 4 days, INF-γ levels of lacrimal glands were higher in the TM group (0.34 ± 0.04 pg/μg total protein) compared with the sham (0.23 ± 0.08 pg/μg total protein) or the vehicle group (0.24 ± 0.07 pg/μg total protein) (P = 0.034 and 0.024, respectively), but were not different between the TM and RM5 groups (0.28 ± 0.04 pg/μg total protein in RM5 group, Fig. 5E). Immunohistochemistry revealed that IL-6 and INF-γ expression in lacrimal glands increased in the TM group. At 1 week, INF-γ levels of lacrimal glands were elevated in the TM group (0.45 ± 0.10 pg/μg total protein) compared with the sham (0.23 ± 0.05 pg/μg total protein) or the vehicle group (0.23 ± 0.05 pg/μg total protein) (P = 0.002 for both comparison, respectively), and were reduced by 50% in the RM5 group (0.50 ± 0.06 pg/μg total protein) compared with the TM group (P = 0.021). At 2 weeks, INF-γ levels of lacrimal glands were higher in the TM group (0.37 ± 0.05 pg/μg total protein) compared with the sham (0.24 ± 0.03 pg/μg total protein) or the vehicle group (0.23 ± 0.03 pg/μg total protein) (P < 0.001 for both comparison, respectively), but were 27% lower in the RM5 group (0.27 ± 0.05 pg/μg total protein) compared with the TM group (P = 0.025). Protein expression in lacrimal glands was evaluated using Western blot. Expression of cleaved ATF6 (cATF6) increased in the TM group compared with the sham or the vehicle group or the RM5 group at all time points (Fig. 5F). Expression of cleaved ATF6 (cATF6) was 80% and 74% lower in RM5 group (93.86% ± 20.51% at 1 week and 104.38% ± 81.95% at 2 weeks) compared with the TM group (475.08% ± 136.75% at 1 week and 409.0% ± 116.35% at 2 weeks) at 1 week and 2 weeks (P = 0.014 and 0.016, respectively). XBP-1, CHOP, and GRP78 expressions were increased in the TM group (571.67% ± 202.21%, 523.27% ± 112.08%, and 382.68% ± 124.50%) compared with the sham and reduced by 77%, 64%, and 62% in the RM5 group (153.69% ± 53.34%, 189.50% ± 73.27%, and 143.89% ± 28.31%) compared with the TM group (P = 0.015, 0.003, and 0.018 between the sham and TM group; P = 0.022, 0.013, and 0.051 between TM group and RM5 group, respectively; Fig. 5G).

**Discussion**

Dry eye syndrome is a chronic disease, characterized by a tear film dysfunction and chronic inflammation of the lacrimal glands, ocular surface, and interconnecting innervation. Oxidative stress and ER stress also contribute to the pathophysiology of dry eye syndrome. In this study, tunicamycin, an ER stress inducer, reduced tear production, increased blinking rate, and increased corneal FSS, which are all hallmarks of dry eye syndrome. This study is the first to report dry eye syndrome by inducing ER stress in mice by injection of tunicamycin, different from the previous studies using NOD mice. This study revealed that rapamycin ameliorated this tunicamycin-induced dry eye syndrome.

This study showed that tunicamycin induced ER stress of the conjunctiva and the lacrimal glands in mice. By blocking N-linked glycosylation, tunicamycin induces unfolded proteins to accumulate in the lumen of the ER, a condition known as ER stress. In this study, tunicamycin increased expression of p-PERK and cATF6. The unfolded protein response is activated to depend on three ER-resident sensor proteins: inositol-requiring protein-1α (IRE1α), PERK, and ATF6. PERK is the major protein that attenuates mRNA translation under ER stress, which reduces protein synthesis and inhibits entry of newly made proteins into the stressed ER lumen. In response to ER stress, PERK is phosphorylated to p-PERK, which is used as an ER stress marker. ATF6 is activated on ER stress. The activated form of ATF6 moves into the nucleus, and activates target genes via binding to ER stress response elements. This study showed that the expressions of XBP-1 and GRP78 were increased in the conjunctiva and lacrimal glands of the TM group. XBP-1 and GRP78 are elevated in response to the ER stress. Sustained activation of XBP-1 causes apoptosis and inflammation, although XBP-1 is required for biosynthesis in exocrine glands. It has been reported that XBP-1 and GRP78 in the conjunctiva is induced by IκB-γ in Sjögren syndrome, which is compatible with this study. In this study, CHOP also increased in the lacrimal glands of TM group. CHOP (GADD153/DDIT3), which is activated by ER stress, directs cell fate toward apoptosis. In this study, rapamycin repressed the ER stress-associated proteins, p-PERK and ATF6. Rapamycin is known to inhibit mTOR. The mTOR complex 1 (mTORC1) has been reported to contribute to ER stress–associated apoptosis through activation of the IRE1/c-Jun N-terminal kinases pathway. Inhibition of mTOR suppresses the activation of PERK and CHOP, the ER stress markers.

This study showed that tunicamycin-induced ER stress repressed tear production, reduced the size and PEeCAM-1 expression of lacrimal glands, and increased the levels of angiogenic factors, HIF-1α and VEGF, in lacrimal glands, which were all attenuated by rapamycin. PEeCAM-1 is a molecule expressed on all cells within the vascular compartment. PEeCAM-1 was investigated as a vascular marker because it is expressed in endothelial cells although it is also expressed in platelets and leukocytes. VE-cadherin was evaluated as a specific vascular endothelial cell marker. VE-cadherin was suppressed in the TM group and increased in the RM5 group.
FIGURE 5. Inflammation of lacrimal glands. (A) Hematoxylin and eosin staining showed that acinar atrophy and ductal infiltration of inflammatory cells (mostly neutrophils and macrophages) was present in the TM group. Intact acinar cells and fewer infiltrates of inflammatory cells were observed in the RM5 group compared with the TM group. Magnification ×200. (B) CD11b<sup>+</sup> cells were infiltrated in the TM group. No CD11b<sup>+</sup> cell was detected in the sham or the vehicle group or RM5 group. (C) CD4<sup>+</sup> cells were infiltrated in the TM group. No CD4<sup>+</sup> cell was detected in the sham or the vehicle group or RM5 group. (D) GRP78 expression of lacrimal glands increased in the TM group. (E) IL-6 levels and INF-γ levels of lacrimal glands were evaluated at 4 days, 1 week, and 2 weeks. (F) Expression of cATF6 increased in the TM group. (G) XBP-1, CHOP, and GRP78 expression was increased in the TM group. *Statistically significant between TM group and RM5 group by independent t-test.
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Figure 6. Schematic diagram of connection of ER stress and inflammation.

compared with the TM group. These results suggest that ER stress leads to vascular endothelial damages. Rapamycin inhibited ER stress–induced vascular damage. Decreased vascular density reduces the supply of oxygen and leads to tissue hypoxia. HIF-1α is an essential regulator of vascularization and anaerobic metabolism and is crucial for immunological responses. HIF-1α levels increase during hypoxia and upregulate VEGF. VEGF is an angiogenic factor that increases in response to hypoxia. Tunicamycin-induced ER stress causes vascular damage and capillary dropout, which results in tissue hypoxia. Subsequently, elevation of HIF-1α in response to hypoxia leads to increased VEGF levels. The reduction of vascularity of lacrimal glands may lead to acinar atrophy and decreased tear secretion, resulting in dry eye syndrome. In this study, rapamycin reduced ER stress and protected the vessels of lacrimal glands. Hypoxia promotes vascular endothelial cell proliferation in vitro via an mTOR-dependent pathway. mTOR inhibition by rapamycin ameliorates hypoxia-induced vascular cell proliferation and angiogenesis. In this study, rapamycin increased the LC3-II/LC3-I ratio. LC3-I is converted to LC3-II in an autophagic process. Rapamycin protected the lacrimal glands through inducing autophagy. The AKT pathway is important in cell survival and protects the vessels from damages. In this study, rapamycin upregulated AKT. Thus, rapamycin protected the lacrimal glands from ER stress through upregulation of AKT and induction of autophagy. In this study, the size of lacrimal glands in the RM5 group was larger compared with the TM group. Nevertheless, HIF-1α and VEGF levels in lacrimal glands were not different between the RM5 group and TM group at day 4. The effect of rapamycin may be delayed on HIF-1α and VEGF levels.

In this study, tunicamycin-induced ER stress elevated the IL-6 and IFN-γ levels of the ocular surface and lacrimal glands. Both FSS and blinking rate were increased by tunicamycin-induced ER stress. Histologic examination showed that inflammatory cell infiltration and acinar atrophy in the lacrimal glands were induced by tunicamycin-induced ER stress. This study showed that CD4+ cells and CD11b+ cells were infiltrated in lacrimal glands. CD4+ cells mediate dry eye syndrome and ocular surface inflammation. CD11b+ dendritic cells are important in CD4+ cell activation. IL-6 and IFN-γ are proinflammatory cytokines that increase in vascular dysfunction, vascular disease, and dry eye syndrome. ER stress impairs Toll-like receptor signaling and causes inflammation. ER stress cleaves and activates membrane-bound ATF6. ATF6 activation links ER stress to inflammation. ATF6 mediates TLR activation, which regulates innate immune responses in macrophages. In this study, rapamycin ameliorated ER stress–induced inflammation. Proinflammatory cytokine levels of the ocular surface and lacrimal glands were reduced by rapamycin. Rapamycin ameliorated ER stress–induced inflammatory cell infiltration and acinar atrophy of lacrimal glands, as determined by histological staining. Rapamycin inhibits both the mTOR signaling pathway and the activation of transcription factors, nuclear factor-kB and CREB. Schematic diagram is shown in Figure 6. Vehicle group was injected intraperitoneally with PBS. Intraperitoneal injection of PBS alone does not increase inflammatory cytokines in the lacrimal glands. PBS does not induce inflammation and injury. The group that did not undergo any injection served as control (sham).

We used Balb/c mice, which have been reported to be a Th2 dominant mouse strain and resistant to dry eye. However, Balb/c mice also increased Th1 and Th17 responses. Furthermore, Balb/c mice are susceptible for ER stress and considered to be appropriate to investigate ER-included inflammation. The mice were observed for 2 weeks. Two weeks was considered enough to investigate the effect of TM or RM5 on ER stress–induced inflammation. Long-term use of tunicamycin is potentially lethal. The effect of tunicamycin was the most effective at 2 weeks in a previous study. The limitation of this study is that ER stress inhibitor was not used. Further study is necessary to investigate which specific ER stress inhibitor can reverse the effect of tunicamycin on dry eye syndrome.

In summary, tunicamycin-induced ER stress in lacrimal glands leads to dropout of capillaries and acinar atrophy of lacrimal glands, which reduces tear production and induces dry eye syndrome on the ocular surface. Rapamycin prevents dry eye syndrome by reducing tunicamycin-induced ER stress in lacrimal glands and preserving capillaries and acinar cells in the lacrimal glands. In conclusion, intraperitoneal injection of tunicamycin induces dry eye syndrome in mice. Rapamycin protects the tear and ocular surface against ER stress–induced dry eye syndrome in mice by ameliorating ER stress–induced vascular damage and inflammation of the ocular surface and lacrimal glands.

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