Impact of Repeated Topical-Loaded Manganese-Enhanced MRI on the Mouse Visual System

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PURPOSE. Optic nerve degeneration in diseases such as glaucoma and multiple sclerosis evolves in months to years. The use of Mn²⁺-Enhanced Magnetic Resonance Imaging (MEMRI) in a time-course study may provide new insights into the disease progression. Previously, we demonstrated the feasibility of using a topical administration for Mn²⁺ delivery to the visual system. This study is to evaluate the impact of biweekly or monthly repeated Mn²⁺ topical administration and the pH levels of the Mn²⁺ solutions for MEMRI on the mouse visual pathway.

METHODS. Using groups of mice, the MEMRI with an acidic or pH neutralized 1 M MnCl₂ solution was performed. To evaluate the feasibility of repeated MEMRIs, topical-loaded MEMRI was conducted biweekly seven times or monthly three times. The enhancement of MEMRI in the visual system was quantified. After repeated MEMRIs, the corneas were examined by optical coherence tomography. The retinal ganglion cells (RGCs) and optic nerves were examined by histology.

RESULTS. All mice exhibited consistent enhancements along the visual system following repeated MEMRIs. The acidic Mn²⁺ solution induced a greater MEMRI enhancement as compared with a neutral pH Mn²⁺ solution. Significant 20% RGC loss was found after three biweekly Mn²⁺ inductions, but no RGC loss was found after three monthly Mn²⁺ treatments. The corneal thickness was found increased after seven biweekly topical-loaded MEMRI.

CONCLUSIONS. Acidic Mn²⁺ solutions enhanced the uptake of Mn²⁺ observed on the MEMRI. Increasing the time intervals of repeated Mn²⁺ topical administration reduced the adverse effects caused by MEMRI. (*Invest Ophthalmol Vis Sci.* 2012; 53:4699–4709) DOI:10.1167/iovs.12-9715

Mn²⁺ enhanced Magnetic Resonance Imaging (MEMRI) is a noninvasive imaging modality to explore the structural and functional characteristics in the central nervous system in animals.^{1–4} In the visual system, an intraperitoneal administration of Mn²⁺ allowed the MEMRI to characterize the ion channel regulation in photoreceptors^{5,6} and retinal layer-specific functionality.⁷ After an Mn²⁺ intravitreal injection, Mn²⁺ was absorbed into the retinal ganglion cells (RGCs),

transported along the optic nerves,^{8,9} and distributed to the superior colliculus and visual cortex.^{2,7,10–13} MEMRI offered a noninvasive approach to characterize retinotopic mapping, which was shown to reflect the optic nerve damage as well the neuroplasticity for vision development.^{10,11,14} Following an Mn²⁺ intravitreal injection, the MEMRI has been demonstrated to be particularly usable for monitoring degeneration and repair of the optic nerve.^{10,15–18} MEMRI has also been used to investigate axonal transport deficit caused by microtubule disruption or oxidative stress.^{19–22} Dynamic imaging following a single Mn²⁺ intravitreal injection may provide a quantitative evaluation of axonal transport in optic nerves, which may provide new insights to RGC damage involved in glaucoma or other visual diseases.^{8,9,23}

We previously explored the use of a topical administration as an alternative to the intravitreal injection to deliver Mn²⁺ to the mouse visual system.²⁴ We demonstrated that following a drop of 1 M MnCl₂, significant signal increments were found in the retina, optic nerves, lateral geniculate nucleus, and the superior colliculus on T1-weighted images. The signal reached the peak in 1 day and returned to the baseline within 7 days. Immunohistochemistry confirmed that the topical administration of MEMRI did not cause retinal and optic nerve damage. Compared with the traditional intravitreal injection, the topical loading approach avoids any chance of causing subconjunctival hemorrhage or trauma to the sclera. The topical loading approach may improve the feasibility of MEMRI in a time-course study to monitor the disease process or development in the visual system.

We have noticed that though the Mn²⁺ solution appeared at a neutral pH value when it was just made, the solution gradually becomes acidic. As such, one goal of this study was to examine the effect of pH on MEMRI. Although a neutral pH might be preferred for biological applications, many therapeutic topical ophthalmic solutions, such as cyclopentolate, tropicamide, and ciprofloxacin, have a pH value of 4–5. In this study, we conducted MEMRI with a fresh prepared solution (pH of 7.4) for the topical loading. To evaluate the effects of a low pH solution on MEMRI, we also reduced the pH of the freshly prepared Mn²⁺ solution by adding HCl before the topical administration. We also performed MEMRI using 1- or 5-day-old Mn²⁺ solutions (pH of 5) with or without pH neutralization before the topical administrations.

Previous studies have demonstrated the safety of applying MEMRI to the visual nervous system following a single intravitreal injection^{2,7,10–13} or a single topical administration.^{24–27} Diseases such as glaucoma and multiple sclerosis are featured with optic nerve degeneration evolving in months to years. In the case of the animal model of multiple sclerosis, the experimental autoimmune encephalomyelitis, the iterations of relapsing and remitting phases, can proceed in a period of 3 months before reaching a stabilized status.²⁸ We used this disease model to examine the feasibility of MEMRI in a time-course study of biweekly (once every 2 weeks) or monthly topical administration of Mn²⁺ over 3 months. Signal enhance-

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FIGURE 1. T1WI of a normal mouse 1 day after 1 M Mn²⁺ topical administration. Signal enhancement was seen in the right retina (a), right optic nerve (b, c), left lateral geniculate nucleus (d) and the left superior colliculus (e, f) 1 day after a topical loading of 1 M MnCl₂ on the right eye. Regions of interest were selected from the retina (~80 voxels, on the image slice with the middle section of an eye, g), optic nerve (3 × 3 voxel square, ~1.5 mm anterior to chiasm, h), and superior colliculus (~25 voxels, i).

ments along the visual pathway were determined using MEMRI following each application. To evaluate the integrity of eye tissues following repeated MEMRIs, optical coherence tomography (OCT) was performed to examine the corneal integrity. Immunohistochemistry was performed to assess the RGCs in the retina and their axons in the optic nerves.

MATERIALS AND METHODS

All animal procedures were done in accordance with National Institutes of Health guidelines and the Statement for the Use of Animals in Ophthalmic and Visual Research, and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Mn²⁺ Topical Loading

Female C57BL/6 mice at 8 weeks old were anesthetized by 1.5% isoflurane/oxygen using an isoflurane vaporizer (VetEquip, Pleasanton, CA). The body temperature was maintained using an electric heating pad; 5 µL MnCl₂ was administered to the surface of the right eye of each anesthetized mouse. After 1 hour, the remaining solution was carefully removed by lint-free tissue (Kimwipes; Kimberly-Clark, Ontario, Canada). Mice were then released to their original cages.

Mn²⁺ Solution Preparation, Experimental Groups, and Time Course

pH Effects. Four groups of mice were used (*N* = 5 each). Two groups of mice received 1 M MnCl₂ mixed in distilled and deionized water (dH₂O) prepared 1 and 5 days before the topical administration. Both solutions appeared with a similar pH level of 4.5–5 before the topical loading. The other two groups received these solutions with pH neutralization before the topical administrations. MRI was conducted 1 and 7 days after each topical loading. To evaluate the reproducibility of these approaches, the entire procedure was repeated three times biweekly in each animal.

Using another group of mice (*N* = 5), 1.0 M MnCl₂ in dH₂O was freshly prepared for the topical loading. While the solution appeared with a neutral pH, HCl was added to reduce the pH to 5 before the topical administration. MRI was conducted 1 day after the topical loading. The entire procedure was repeated three times biweekly in each animal. Data were compared with the MEMRI from a freshly prepared solution (*N* = 5).

Repeated MEMRIs. Four groups of mice were used in seven biweekly (once in every 2 weeks) repeated MEMRIs (*N* = 6 each). 1 M MnCl₂ solution was prepared with variations: mixed in 0.01 M PBS and saline (group 1) or dH₂O (group 2) 1 day before the topical loading. For group 3, the pH level of the 1.0 M MnCl₂ in dH₂O was adjusted to a neutral level before the topical loading. For group 4, 1.0 M MnCl₂ in dH₂O was prepared immediately before the topical administration. MRI

FIGURE 2. Biweekly MEMRI using solutions prepared 1 or 5 days before the topical loadings (pH = 5) with or without pH adjustment. All Mn^{2+} -affected regions of interest showed significant enhancements as compared with the control sites (paired *t*-test). The bracket indicates $P < 0.05$ via multiple comparisons between groups (*t*-test). When data (a–c) was rearranged based on the pH level (d), MEMRI with a low pH solution showed greater enhancement as compared with the MEMRI with a neutralized pH solution (*t*-test).

was conducted at day 1 after the topical loading. The entire procedure was repeated seven times biweekly on each animal.

MRI Procedure

Mice were anesthetized by 1.5% isoflurane/oxygen using an isoflurane vaporizer (VetEquip) for imaging. The core body temperature was maintained using a warm water circulating pad. A 7-cm inner diameter Bruker linear RF coil was used as a transmitter, and a 2-cm surface coil was used as a receiver. T1-weighted spin-echo image (T1WI) was taken using a Bruker 4.7T BioSpec animal scanner with TR of 380 ms, TE of 8.5 ms, 32 averages, field of view of 1.5 cm, slice thickness of 0.5 mm, and data matrix of 96×96 (zero-padding to 256×256). Nineteen contiguous transactional slices were selected to cover the visual system from the eye to the superior colliculus. The total scanning time was 20 minutes.

Regions of interest (ROIs) were selected from the retina, prechiasmatic optic nerves, and superior colliculus from left and right hemispheres. An example of ROI is shown in Figure 1. The signal intensity of the Mn^{2+} -affected site was divided by the signal intensity measured from the same anatomical region from the opposite hemisphere. Data were presented as mean $\pm$ standard deviation. Repeated measures analysis of variance (ANOVA) was carried out followed by the Bonferroni-adjusted *t*-test with $P < 0.05$ considered to be statistically significant. Statistical analysis was conducted using data

analysis and graphing software (SigmaPlot 11; Systat Software Inc., San Jose, CA).

OCT Procedures

At the end of the MEMRI time-course evaluation, the spectral domain OCT (SD-OCT; Biopogen Inc., Research Triangle Park, NC) was used to examine the integrity of the cornea of each mouse. Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine hydrochloride and 10 mg/kg xylazine drug mixture. Pupils were dilated using a topically applied drop of tropicamide (1%; Falcon Pharmaceuticals, Fort Worth, TX). Corneas were lubricated frequently during the imaging session (Systane Ultra ophthalmic lubricant; Alcon Ltd., Fort Worth, TX). Volumetric images were acquired with 1000 A-scans per B-scan, 100 B-scan frames, and 1024 samplings/A-scan in depth. Four repeated A-scans were collected and averaged at each location to improve signal-to-noise ratio. This corresponds to a volume of approximately $6 \times 6 \times 1.14$ mm.

Immunohistochemistry Examination

All mice from the longitudinal MEMRI study were sacrificed for immunohistochemistry to examine the integrity of retina and optic nerves. In addition, 24 female C57BL/6 mice at 8 weeks old received $5 \mu L$ 1.0 M $MnCl_2$ topical loading biweekly (1.0 M $MnCl_2$ in dH_2O fresh prepared right before the topical administration), and animals were

FIGURE 3. Enhancements at 12 and 24 hours after the topical loading using acidic Mn^{2+} solution with or without pH neutralization. The signal enhancement at these two time points were similar to each other with a $\sim 10\%$ increase in enhancement in the superior colliculus of the low pH group and a $\sim 5\%$ decrease in enhancement of optic nerves at 24 hours, compared with the 12-hour measurements. $*P < 0.05$ with a paired *t*-test.

sacrificed the next day after two, three, four, and five times of MnCl_2 topical loading with $N = 6$ at each time point. Because we found a significant 30% RGC loss after three times of biweekly MEMRI, to evaluate the possible delayed damage from the first two times of biweekly MEMRI, another group of mice ($N = 6$) received two biweekly MEMRI and were sacrificed in 4 weeks after the last MEMRI. To evaluate the effects of extending the time intervals of the repeated MEMRIs, using another group of mice, animals received repeated monthly topical administration of 1.0 M MnCl_2 . Six mice were sacrificed in 1 month after two times of monthly topical administration, and six mice were sacrificed after three times of monthly topical administration of 1 M MnCl_2 for histology analysis.

For immunohistochemistry examination, animals were perfusion fixed. The perfusion fixing was achieved by injecting the left cardiac ventricle with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS 1 week after Mn^{2+} treatment. A 4-mm-thick coronal section (-1 to $+3$ mm of bregma) was obtained from each brain

and embedded in paraffin. Tissue slices (3 μm thickness) of optic nerves, ~ 1.5 mm anterior to chiasm, matching the MRI (Fig. 1) were cut and deparaffinized in xylene for immunohistochemical examinations. The integrity of axons was evaluated using a primary antibody against nonphosphorylated neurofilament (SMI-31, 1:1000; Sternberger Monoclonals, Lutherville, MD).²⁹ Following a 15-minute wash in PBS, sections were incubated in fluorescent secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG, 1:200, Carlsbad, CA) for 1 hour at room temperature. In addition, H&E staining was done on the eye tissue of the 1.5 MnCl_2 -treated mice to evaluate the RGC.

Histological sections were examined using a confocal microscope (Olympus FluoView; Olympus Corp., Center Valley, PA) equipped with a 120 $\times$ oil objective. The green SMI-31 positive staining, representing the normal axons, was captured. Axons were counted through the central $70 \times 70 \mu\text{m}^2$ regions. The counts were presented as mean $\pm$ standard deviation. For RGC evaluation, the middle section of the eyeball was used to represent the RGC integrity of each eye. The tissue

FIGURE 4. Biweekly MEMRI with a fresh prepared Mn^{2+} solution with HCl added to reduce the pH compared with the MEMRI with a fresh prepared solution (pH of 7.4). All Mn^{2+} -affected regions, including the retina, optic nerves, and superior colliculus, showed significant enhancement as compared with the non- Mn^{2+} affected controls (paired *t*-test). Although the difference did not reach a statistic significance level, the low pH solutions led to a higher enhancement in MEMRI as compared to those with a neutral pH solution.

section was examined with a 40 $\times$ objective. Eight pictures were taken to cover the entire RGC layer to quantify the RGC cell density. A two-tailed *t*-test was performed to compare the measurements between control and Mn^{2+} -affected eyes.

RESULTS

MEMRI with a 1- or 5-Day-Old Solution with pH 5 or 7.4

We observed that the pH value of the Mn^{2+} solution appeared as 7.4 when the solution was made. The pH decreased to 4.5–5 in a day and remained at this level over the next 5 days. To determine if the pH of the Mn^{2+} solution affected the degree of MEMRI enhancement, four groups of mice were used: group 1 received a 1 M Mn^{2+} solution 24 hours after preparation with a pH = 5; group 2 received a 1 M Mn^{2+} solution 24 hours after preparation where the pH was adjusted 7.4; group 3 received a 1 M Mn^{2+} solution 5 days after preparation with a pH = 5; and group 4 received a 1 M Mn^{2+} solution 5 days after preparation where the pH was adjusted to 7.4. MRI was performed at 1 and 7 days after the topical loading. All procedures were repeated three times biweekly. In 1 day after each topical loading, all groups of mice showed significant enhancement along the visual pathway, including the right retina, right optic nerves, left lateral geniculate nucleus, and left superior colliculus (Fig. 1). Compared with the intensity without Mn^{2+} , significant increments of signal were found (Fig. 2) in the retina (40%–60% increments, $P < 0.05$); in the optic nerves (20%–30% increments, $P < 0.05$); and in the superior colliculus (5%–15% increments, $P < 0.05$). The repeated MEMRIs did not show significant difference as compared with their first MEMRI. To confirm the Mn^{2+} clearance before the next topical loading, MRI was also performed at 7 days after each topical loading. We found that all measured signals returned to the control level with no significant difference compared with the no- Mn^{2+} treated images. Between group comparisons showed that (Fig.

2), in retina, in the second MEMRI, the 1-day-old, pH 7.4 solution showed ~30% lower enhancement than other groups. In optic nerves, in the second MEMRI, 5-day-old pH 5 solution showed a ~10% lower enhancement compared with the 1-day-old pH 5 solution. In the third MEMRI, pH 7.4 solutions showed 5%–10% lower enhancements in optic nerves than the pH 5 solutions. In the superior colliculus, in the third MEMRI, the 1-day-old pH 7.4 solution showed a significant ~10% lower enhancement than the 5-day-old pH 5 solution.

When animals were grouped according to the pH of the solution used, the MEMRI using the solution with an adjusted pH (7.4) exhibited lower enhancement in all ROIs, compared with the low pH solution groups, including retina (1.6 vs. 1.5, $P = 0.061$); optic nerves (1.24 vs. 1.16, $P = 0.005$); and superior colliculus (1.12 vs. 1.08, $P = 0.076$). To better understand if solutions with different pH levels affect Mn^{2+} uptake and clearance rates, we randomly picked 12 mice (3 mice from each group), and performed the MRI at 12 and 24 hours after topical loading. As shown in Figure 3, the level of enhancement measured at 12 hours was relatively equivalent to the level of enhancement at 24 hours. Subtle but significant differences were found in the superior colliculus of the pH 5 groups (~10% increase at 24 hours) and in the optic nerves of the pH 7.4 groups (~5% decrease at 24 hours).

MEMRI Using the Fresh Prepared Solution Added with HCl to Reduce the pH to 5

To further examine the effects of using a low pH Mn^{2+} solution in MEMRI, the freshly prepared Mn^{2+} solution was added with HCl to reduce the pH from its initial 7.4 to 5. MRI was performed in a day after the topical loading ($N = 5$). The topical loading and MRI were repeated biweekly three times. Data was compared with the MEMRI using a freshly prepared Mn^{2+} solution (pH = 7.4, $N = 5$). As shown in Figure 4, all groups of mice showed significant enhancements in retina, optic nerves, and superior colliculus in 1 day after each topical loading.

FIGURE 5. Enhancements of retina, optic nerve (ON), and superior colliculus (SC) following biweekly MEMRI. All measured signals from first-seventh MEMRI exhibited significant enhancements compared with the non-Mn²⁺-affected controls ($P < 0.05$, paired t -test). When we compared the first of 7 biweekly MEMRI between groups 1–4, only the retinas between group 2 and group 3 showed a significant difference ($P < 0.05$, multiple comparison t -tests). When we compared to the first MEMRI, the only difference was found in the retina of group 3 at the seventh MEMRI. * $P < 0.05$, multiple comparison t -tests.

There was no statistical difference between groups or between time points. However, the pH 5 group consistently (at all three time points) showed higher enhancements in optic nerves and superior colliculus as compared with the pH 7 group, although these differences did not reach statistical significance.

Biweekly Repeated MEMRIs for 14 Weeks

Four groups of mice received MEMRI biweekly over a period of 14 weeks. As summarized in Figure 5, all animals showed significant signal enhancement in the retina, optic nerves, and superior colliculus at each time point. In all measured ROIs,

FIGURE 6. Cornea OCT after seven biweekly MEMRI. The thickness of cornea was measured from Mn²⁺-affected corneas and non-Mn²⁺ controls. All groups of Mn²⁺-affected corneas showed significantly increased thickness ($P < 0.05$, paired t -test). The top four thickest corneas (circled) also appeared with increased opacity, which can be observed as white eyes in these mice.

the signal enhancement did not vary over time with repeated MEMRIs, compared to the first MEMRI, with the exception of the retina in group 3, where a significant increase in enhancement was seen in the seventh MEMRI. It was worth noting that mice in the Group 2 showed a relatively higher enhancement than other groups, with retinal enhancement reaching a statistical significant level, compared with group 3.

Corneal and Retinal Damage

Following the final MEMRI, OCT was used to study the cornea and anterior segment of the eye. As shown in Figure 6, the Mn²⁺-affected corneas were consistently thicker than the nontreated (control) corneas. The thickening ratios varied in a range from ~10% to 300% among animals. The four thickest corneas were associated with a noticeable increase of opacity, which can also be identified as white coloring eyes in these animals (Fig. 6). While OCT is also a powerful tool to examine the retina, we were unable to perform consistent measurements of the retinal integrity of these animals due to the opaque cornea in some animals.

The RGC integrity was examined using H&E histological staining to examine the RGC cell bodies in the retina (Fig. 7). Mice were sacrificed for retinal histology after 2–7 biweekly Mn²⁺ topical administrations or after 2 and 3 monthly Mn²⁺ topical administrations, respectively ($N = 6$ per group). Retinal histology showed that RGC integrity remained normal after the second biweekly, and after 2 and 3 monthly Mn²⁺ treatments (Fig. 7). We also examined the RGC in mice sacrificed at 4 weeks after two times of biweekly Mn²⁺ treatments. As shown in Figure 7, there is no significant RGC loss in this group of mice, suggesting no delayed damage. However, after the third biweekly Mn²⁺ treatment, a significant 20% loss of RGC was measured. Following the biweekly treatment groups, retinal histology showed a significant 30%–40% loss of RGC after four or five Mn²⁺ treatments (Fig. 7), suggesting that accumulated treatment with Mn²⁺ leads to more severe RGC damage.

After seven biweekly MEMRI, we also used SMI-31 antibody to detect the RGC axonal density in optic nerves (Fig. 8). The SMI-31 immunohistochemical staining showed abnormal axons in optic nerves (Fig. 8). Quantitative analysis showed a

FIGURE 7. H&E staining analysis of Mn²⁺ affected mice. H&E staining (a) shows significant loss of RGC after repeated MEMRIs. Quantitative analysis shows RGC density gradually decreased following two, three, four, five, and seven biweekly Mn²⁺ treatments (b). **P* < 0.05 (*t*-test) compared with the control (Ctr, mice without Mn²⁺ treatments). Grp 1–Grp 4 refers to the Mn²⁺ solutions used for MEMRI. Grp 1: Mn²⁺ in saline (1 day old); Grp 2: Mn²⁺ in dH₂O (1 day old); Grp 3: Mn²⁺ in dH₂O with NaOH to adjust pH; and Grp 4: freshly prepared Mn²⁺ in dH₂O.

significant 30% loss of SMI-31 positive axons in the mice of group 1. Although mice in the other groups did not show significant axonal loss, swollen and irregular axonal formations suggested mild damage to the optic nerve axons resulting from repeated MEMRIs (Fig. 8B).

DISCUSSION

Previously, we demonstrated that topical administration can serve as an alternative to an intravitreal injection for Mn²⁺ delivery into the visual system for MEMRI.²⁴ One day after the Mn²⁺ topical loading, significant enhancements on the T1-weighted images were seen in the retina, optic nerves, and superior colliculus. The enhancements returned to the baseline in a week with no detectable damage to the eye and optic nerves. Because the topical loading approach avoids the need to perform the traditional intravitreal injection,^{2,7,10–13} the topical loading approach improves the feasibility of using MEMRI repeatedly in a time-course study. This study is to

evaluate the feasibility of repeated Mn²⁺ topical administration and the pH levels of the Mn²⁺ solutions for MEMRI on the mouse visual pathway. It was observed that the Mn²⁺ solution with a low pH value rendered stronger MEMRI enhancements. Repeated MEMRIs produced consistent enhancements with no detectable retinal damage after two biweekly or three monthly applications.

The Effect of Storage Length of Mn²⁺ Solution and pH Effects on MEMRI

We observed that the freshly made Mn²⁺ solution had a pH of 7.4, and within 1 day became acidic (pH of 4.5–5; sealed, stored in room temperature).³⁰ While both the 1- and 5-day-old solutions had similar pH levels of 4.5–5, the MEMRI of 1-day-old solution generally had a higher enhancement than that of the 5-day-old solution, although the differences did not reach statistical significance (except in the optic nerve of the second MEMRI). We speculated that the longer storage time may give a higher chance of producing Mn²⁺-related precipitation, so the 1-day solution had a higher Mn²⁺ uptake than the 5-day solution. Because of the low pH values of these solutions, we also tested MEMRI pH with neutralized solutions. We observed that, as shown in Figure 2, the MEMRI with pH neutralized solutions exhibited a lower enhancement in all regions of interest including retina (1.6 vs. 1.5, *P* = 0.061); optic nerves (1.24 vs. 1.16, *P* = 0.005); and superior colliculus (1.12 vs. 1.08, *P* = 0.076). The reasons for a low pH solution to affect the enhancements of MEMRI may be complicated. Multiple factors may be involved to cause the low pH solution to enhance the Mn²⁺ uptakes. It is possible that through pH neutralization, the amount of soluble Mn²⁺ may be reduced leading to a decreased Mn²⁺ concentration in the solution used for topical loading. It is also known that low pH solutions can promote vasodilation,^{31,32} which may increase tissue permeability, resulting in a higher Mn²⁺ uptake in the visual system. In our other experiments, the freshly prepared Mn²⁺ solution was added with HCl decreasing its pH from 7.4 to 5 before the topical loading. We observed that the acidic solution increased the enhancements, especially in optic nerves (1.24 vs. 1.13) and superior colliculus (1.17 vs. 1.05), as shown in Figure 4. Collectively, all these data support that low pH enhances the Mn²⁺ signal in MEMRI.

Seven Biweekly MEMRIs

Because MEMRI has the unique capability to provide both structural and functional information of the neural system,^{1–4} the use of MEMRI in a time-course study can provide new insights into disease progression. In diseases such as glaucoma, multiple sclerosis, and Alzheimer's disease, neurodegeneration usually takes months to years.^{8,9,23,28,33} Our previous study, using an animal model of multiple sclerosis, showed that the relapsing and remitting iterations along with the neurodegeneration induced by optic neuritis may proceed for up to 3 months.²⁸ Given the advantages of the noninvasive topical administration method in this study, we examined the feasibility of performing biweekly or monthly topical loaded MEMRI for a period of 3 months. As shown in Figure 5, we found a relatively consistent enhancement across the repeated performance of MEMRI. All of the repeated MEMRIs showed enhancements with no significant difference as compared with their first MEMRI, except the RGC signal of the seventh MEMRI in group 3.

It is worth noting that group 2 showed higher mean enhancements than the other groups (Fig. 5). Mice in group 2 received a Mn²⁺ solution in dH₂O made 1 day prior to topical loading. The solution was more acidic than the solutions used

FIGURE 8. SMI-31 positive axons in optic nerves of controls (Ctr) and mice after seven times of MEMRI (groups 1–4). SMI-31 was used to stain for normal axons. Quantitative analysis (**D**) showed a significant 30% loss ($*P < 0.05$, *t*-test) of axons in group 1 mice (Grp1), but the counts did not show a statistically significant difference among Groups 2–4 (Grp2–Grp4). Representative staining pictures show that axons in Mn^{2+} treated mice may appear swollen (**B**, a representative picture taken from a Grp2 mouse) or significantly lost (**C**, a representative picture taken from a Grp1 mouse) compared with the control (**A**). The black bar by (**A**) indicates 30 μm .

for the other three groups of mice. The results shown in Figure 5 are consistent with those in Figures 2 and 4 to support that low pH topical Mn^{2+} solutions enhances the Mn^{2+} signals in MEMRI.

Cornea Thickness and Transparency Affected by Repeated MEMRIs

This study also demonstrated that repeated topical loading of Mn^{2+} can cause corneal abnormalities. We found that all Mn^{2+} -affected corneas were ~10% to ~4-fold thicker than normal corneas (Fig. 6). Four of the thickest corneas also showed severe cornea opacity, which also appeared macroscopically as white coloring of the animals' eyes (Fig. 6). The average thickness of the control corneas in this study was ~0.1 mm, which is consistent with measurements taken from naïve mice by other research groups.^{34–36} The cornea, located in the front of the eye, may be more vulnerable to the high concentration of Mn^{2+} , compared with other parts of the eye. It is also possible that repeated topical administration may cause the corneal abnormality. It is also not known whether the repeated use of lint-free tissue, used to remove the remaining solution on the eye after topical administration, irritated the cornea.

The relationship between corneal damage and Mn^{2+} toxicity remains an area in need of further investigation.

Retinal Damage after Repeated MEMRIs

In addition to the corneal damage, the Mn^{2+} -affected eyes also showed a significant 40% RGC loss after seven biweekly repetitions of MEMRI, regardless of the variations in the solutions used in each group of mice. Despite the significant loss of the RGC cells, MEMRI continued to demonstrate enhancements in the retina (Fig. 5). Studies have found that enhanced MEMRI occurred in the brain areas suffering from oxidative stress and gliosis.^{37–39} Thus, it is possible that the pathological events in the injured retina may have enhanced the Mn^{2+} uptake even with a significant loss of neurons. Using another cohort of animals, we found that the toxic effect of Mn^{2+} on RGCs was dependent on the number of repeated inductions. As shown in Figure 7, mice receiving biweekly Mn^{2+} treatments showed a gradual increase in RGC loss: 20% loss (3 times); 30% loss (4 times); and a 40% loss (7 times). When we increased the time intervals from 2 weeks to 1 month, no noticeable RGC damage was observed after two or three times of monthly Mn^{2+} treatments (Fig. 7). The potential neurotoxicity has been a major concern in using MEMRI in neurological studies.^{40–43} Our study suggested that rapidly

repeating Mn^{2+} treatments may increase the risk of MEMRI-induced tissues damage.

Despite the significant loss of RGC in retina, the SMI-31 immunostaining of the optic nerves did not show an equivalent amount of RGC axon loss after repeated MEMRIs. The reason for the discrepancy in retinal and optic nerve damage severity remains unclear. One possible explanation is the damage to the RGCs in the retina has not yet propagated to their distal axons located in the optic nerves. As shown in Figure 8, although the optic nerves did not show severe axonal loss, the immunostaining showed swollen and irregular formation of axons, suggesting a delayed degeneration resulting from RGC loss (Fig. 8B). Additionally, despite the well-known toxic effects of Mn^{2+} ,^{40–43} recent studies have also found that Mn^{2+} may have beneficial effects to injured cells,^{44–46} for instance, via the superoxide scavenging properties of Mn^{2+} to minimize the oxidative stress in the degenerative nerves.^{44,45} It is also possible that exogenous Mn^{2+} may ease the glutamate excitotoxicity in neural diseases.^{45,46} Thus, the possibility of a beneficial role that the low amounts of Mn^{2+} plays in delaying axonal degeneration within the optic nerves may not be excluded.

MEMRI Quantification

Instead of using the signal normalization to quantify the enhancements on MEMRI as performed in this study, a T1 measurement would enhance the quantification of Mn^{2+} uptake in this study. However, to acquire multiple sample points for the curve fitting, a lengthened acquisition time is required. Given the number of experimental groups and the scanning samples per group in this study, we chose to acquire T1-weighted imaging and normalize the T1-weighted signals to internal references. In our previous study, we tested the use of a water tube as an external reference or a signal from fat, muscle, or nonvisual brain region as an internal reference.²⁴ We found that the signal from the septal area provided as a feasible reference, showing the equivalency of the normalized signals from control-retina, optic nerves, and superior colliculus among the scans.²⁴ However, considering the use of a surface coil as a receiver placed on the top of the head, it is possible that the variation of relative horizontal positions between the reference and the targeted regions could add inconsistencies to the signal normalization. Although the B1 falls off gradually in regions away from the coil, the signal intensity is relatively equivalent at a given horizontal level. In a visual pathway, the left and right sites were imaged together at the same horizontal level. Given that the Mn^{2+} was only applied to one eye, leaving the other eye as a control, the signals of the same anatomical regions from the opposite hemisphere served as the reference signals for each region of interest in this study.

In conclusion, this study tested various preparations of Mn^{2+} solution used in the topical-loaded MEMRI. It was found that although the solution exhibited neutral pH when it was first made, it gradually became acidic. The Mn^{2+} solution with a low pH value had a greater enhancement on the MEMRI, compared with the neutral pH solution. Repeated MEMRIs were feasible and produced consistent enhancements. There was no detectable retinal damage after two biweekly or three monthly repetitions of topical-loaded MEMRI.

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