Ability of Mn\(^{2+}\) to Permeate the Eye and Availability of Manganese-enhanced Magnetic Resonance Imaging for Visual Pathway Imaging via Topical Administration

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Abstract

Background: Manganese-enhanced magnetic resonance imaging (MEMRI) for visual pathway imaging via topical administration requires further research. This study investigated the permeability of the corneal epithelium and corneal toxicity after topical administration of Mn\(^{2+}\) to understand the applicability of MEMRI.

Methods: Forty New Zealand rabbits were divided into 0.05 mol/L, 0.10 mol/L, and 0.20 mol/L groups as well as a control group (\(n = 10\) in each group). Each group was further subdivided into epithelium-removed and epithelium-intact subgroups (\(n = 5\) in each subgroup). Rabbids were given 8 drops of MnCl\(_2\) in 5 min intervals. The Mn\(^{2+}\) concentrations in the aqueous and vitreous humors were analyzed using inductively coupled plasma-mass spectrometry at different time points. MEMRI scanning was carried out to image the visual pathway after 24 h. The corneal toxicity of Mn\(^{2+}\) was evaluated with corneal imaging and pathology slices.

Results: Between the aqueous and vitreous humors, there was a 10 h lag for the peak Mn\(^{2+}\) concentration times. The intraocular Mn\(^{2+}\) concentration increased with the concentration gradients of Mn\(^{2+}\) and was higher in the epithelium-removed subgroup than that in the epithelium-intact subgroup. The enhancement of the visual pathway was achieved in the 0.10 mol/L and 0.20 mol/L epithilum-removed subgroups. The corresponding peak concentrations of Mn\(^{2+}\) were 5087 ± 666 ng/ml, 22920 ± 1188 ng/ml in the aqueous humor and 884 ± 78 ng/ml, 2556 ± 492 ng/ml in the vitreous body, respectively. Corneal injury was evident in the epithelium-removed group and was higher in the epithelium-removed subgroup than that in the epithelium-intact subgroup.

Conclusions: The corneal epithelium is a barrier to Mn\(^{2+}\), and the iris and lens septum might be another intraocular barrier to the permeation of Mn\(^{2+}\). An elevated Mn\(^{2+}\) concentration contributes to the increased permeation of Mn\(^{2+}\), higher MEMRI signal, and corneal toxicity. The enhancement of the visual pathway requires an effective Mn\(^{2+}\) distribution within the vitreous body.

Key words: Corneal Permeability; Manganese; Manganese-enhanced Magnetic Resonance Imaging; Optic Nerve; Superior Colliculus; Topical; Toxicity; Visual Pathway

Introduction

Manganese-enhanced magnetic resonance imaging (MEMRI) is a powerful imaging technique used in neuroscience and myocardial physiology and function research.\(^3\)\(^4\) MEMRI depends on two important properties of Mn\(^{2+}\): (1) Mn\(^{2+}\) is a paramagnetic element due to its unpaired electrons, which causes a positive T1 magnetic resonance imaging (MRI) enhancement in Mn\(^{2+}\) accumulated tissue, and (2) Mn\(^{2+}\) is an analog of Ca\(^{2+}\) and can therefore enter an excitable cellular body through voltage-gated Ca\(^{2+}\) channels and become integrated into vesicles, which can be transported across microtubules to neuronal synapses, where the

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Contents can be released at presynaptic membranes and taken up at postsynaptic membranes [Figure 1]. Taking advantage of the properties of Mn2+, MEMRI provides an opportunity to visualize activity in the brain and heart, trace neuron-specific connections in the brain, and enhances the brain cytoarchitecture. In the neuro-ophthalmology research field, MEMRI of the visual pathway has attracted a great deal of attention due to the visual pathway’s distinct anatomical structure and convenient accessibility, which make it a favored structure for studies on neuronal survival, axonal regeneration, and synaptic plasticity in vivo. However, the main drawback of using Mn2+ as an MRI contrast agent is its cellular toxicity. Therefore, it is critical to choose an optimal drug delivery route and use the lowest dose possible.

Our previous study demonstrated that an intravitreal injection was an effective route for administering a contrast agent for MEMRI of the visual pathway. However, we found that an intravitreal injection was in some cases accompanied by severe complications, such as traumatic cataract, intraocular hemorrhage, retinal/choroidal detachment, and endophthalmitis. These disadvantages of intravitreal injections have also been reported by other researchers.

To avoid the disadvantages of an intravitreal injection, researchers have attempted to use topical administration as a noninvasive method for MEMRI of the visual pathway. Sun et al. studied MEMRI of the mouse visual system via a topical administration of a high concentration of Mn2+ (1.00 mol/L). Specifically, they noticed that topically administered Mn2+ did not diffuse into the vitreous space. Lin et al. discovered that 0.75 mol/L could also achieve enhancement of the mouse visual pathway. However, they observed that a high concentration of Mn2+ caused severe corneal damage that manifests as unrecoverable corneal edema. We inferred that it would be feasible to alleviate the corneal injury by reducing the concentration of Mn2+. In the present study, three different concentration gradients (0.05 mol/L, 0.10 mol/L, and 0.20 mol/L) were applied for MEMRI of the visual pathway via topical administration. Because the corneal epithelium is one of the key membranes for regulating the access of xenobiotics into the eye, an epithelium-removed subgroup was tested to investigate the role of the corneal epithelium in the topical administration of Mn2+.

This study investigated the permeation of Mn2+ delivered via topical administration as a contrast agent for an MRI. MEMRI signals were detected after topical administration with a relatively low concentration of Mn2+, and the corneal toxicity induced by Mn2+ was evaluated. These findings should be useful for the selection of a safe dose of MnCl2 for MEMRI via topical administration with an effective administration route.

**Methods**

**Animals and groups**

Adult New Zealand rabbits (License Number: SCXK [Peking] 2015-0016) were purchased from Peking University...
Laboratory Animal Centre. The rabbits were housed individually in mesh cages in an air-conditioned animal room (temperature, 23 ± 2°C; relative humidity, 45 ± 10%) with a 12 h light/dark cycle and were allowed access to food and tap water ad libitum. The animals were treated in accordance with the guidelines approved by the Peking University Animal Ethical Committee for animal research and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Forty male New Zealand rabbits (2 kg) were divided into four groups: 0.05 mol/L group (n = 10), 0.10 mol/L group (n = 10), 0.20 mol/L group (n = 10), and control group (n = 10). Each group was subdivided into an epithelium-removed subgroup (n = 5) and epithelium-intact subgroup (n = 5). In the epithelium-removed subgroup, a 7.50 mm diameter sample of the corneal epithelium was removed from the center of the cornea of the right eye to test the permeability of the epithelial barrier. In the epithelium-intact subgroup, the corneal epithelium remained intact. In the control group, the right eye was treated with normal saline. In the experimental groups, the right eye was treated with MnCl₂ solutions.

**MnCl₂ solution preparation and administration**

A 0.20 mol/L stock solution was prepared by dissolving 2.52 g of MnCl₂ (Sigma-Aldrich Co., Steinheim, Germany; CAS no: 7773-01-5; purity: ≥99%) in 100 ml of sterile deionized water at room temperature. The pH of all solutions was adjusted to 6.90. The osmotic pressure was measured, and the results were as follows: 157 ± 5 mOsmol/kg for 0.05 mol/L, 360.80 ± 2.68 mOsmol/kg for 0.10 mol/L, and 677.60 ± 2.88 mOsmol/kg for 0.20 mol/L. A 0.05 mol/L hypotonic solution was calibrated to 294.40 ± 2.07 mOsmol/kg by adding analytically pure sodium chloride (Camycal Biochemistry Co., Georgia, USA; CAS no: 7647-14-5; purity: 99.50%). Osmolalities were measured at 25°C using a Micro-Osmometer model 210 (Fiske Associates Co., Massachusetts, USA).

The rabbits were anesthetized with an intramuscular injection of a cocktail containing xylazine (5 mg/kg; Fengshou industrial Co., Ltd., Shanghai, China, CAS no: 7361-61-7; purity: ≥99%) and ketamine (35 mg/kg; Chinese and Western Pharmacy Co., Ltd., Shanghai, China, national medicine permission number: H31022247). The rabbits were kept warm on a heating pad with circulating water at 37°C. MnCl₂ solutions and normal saline were administered into the conjunctival sac with no outflow using a pipette. Each rabbit was administered 400 μl delivered in 5 min intervals for 40 min duration.

**Time course and permeation of Mn²⁺ in the aqueous and vitreous humors**

Sample collections were carried out on the right eyes of animals in all of the groups at 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h according to a previous report. Samples were analyzed using an Elan DRC-II inductively coupled plasma-mass spectrometer (ICP-MS, PerkinElmer Life & Analytical Sciences, Shelton, Connecticut, USA).

**Manganese-enhanced magnetic resonance imaging scanning protocol**

Twenty-four hours after MnCl₂ topical administration, the rabbits were anesthetized and kept warm as described above. MRI examinations were carried out using a 3-T MRI system (MAGNETOM Trio Tim, Siemens Co., Ltd., Germany) with an 8-channel knee coil. Heads were placed inside the coil in a prone position. Contrast-to-noise ratio (CNR) values were determined as follows: transverse three-dimensional (3D) T1-weighted fast low-angle shot sequence with fat saturation (repetition time = 23.30 ms, echo time = 3.50 ms, flip angle = 20°, average = 2, field of view = 16.90 cm × 15.80 cm, matrix size = 0.15 mm × 0.15 mm, and slice thickness = 0.70 mm). A maximum intensity projection (MIP) was performed (slice thickness = 10 mm; slice distance = 1 mm). The acquisition time for each 3D T1-weighted image set was 4 min.

Three-dimensional images were analyzed using commercially available software (Mean Curve; Siemens Medical Systems). Manually drawn regions of interest (ROIs) were placed in oblique two-dimensional (2D) slices in the 3D volume to quantify the image enhancement of the optic nerve (ON). The 2D slices with the best visibility of the visual pathway from the ON to the superior colliculus (SC) were chosen. ROIs were placed in the optic foramen and in the SC on two sides. The average ROI value was determined after three measurements. A CNR was calculated using the following formula: CNR = 0.655 × (Sₘ - Sₖ)/SD₉O, where Sₘ and Sₖ represent the signal intensities in the ROI of the MnCl₂-enhanced and contralateral nonenhanced zones, respectively, and SD₉O is the mean value of the signal intensities in two ROIs in air.

**Observation of the corneal toxicity of Mn²⁺**

Corneal images of the right eyes were taken 1 day after a topical administration of MnCl₂. An ophthalmic operating microscope and image acquisition system (Topcon OMS-800 operation microscope and Newcomm digital video monitoring system [Beijing Newcomm Technology, Beijing, China]) were used to obtain corneal imaging results.

To observe corneal edema recovery, rabbits were housed in a temperature-controlled room as described above till day 14, at which time corneal images were obtained again. During the waiting time, the rabbits’ eyes were protected by ofloxacin eye ointment (Santen Pharmaceutical Co., Ltd., Suzhou, China) twice per day.

After corneal images were recorded on day 14, all corneas were excised around the corneal limbus and preserved in 4% paraformaldehyde at 4°C for 24 h. Paraffin sectioning and hematoxylin and eosin (HE) staining were performed. Corneal damage was observed and recorded with a Leica DM4000B microsystem (Leica Microsystems Wetzlar GmbH, Hesse, Germany).
Statistical analysis
Data are shown as the mean ± standard error (SE) and analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). ICP-MS results and CNR values were analyzed with a two-way analysis of variance (ANOVA). A one-way ANOVA was used to analyze the experiment and control groups. Student-Newman-Keuls and Games-Howell tests were used depending on the homogeneity of variances. A Pearson’s correlation analysis was used to analyze the relationship between the peak concentration of Mn\(^{2+}\) in the vitreous humor and CNR. Corneal edema percentage differences between the groups were tested with Fisher’s exact test. A value of \(P < 0.05\) was considered statistically significant.

RESULTS

Time course and permeation of Mn\(^{2+}\) in aqueous and vitreous humors
We observed three concentration gradients of Mn\(^{2+}\) (0.05, 0.10, and 0.20 mol/L) at seven time points (0.5, 1, 2, 4, 8, 12, and 24 h). The concentration of Mn\(^{2+}\) in the aqueous humor reached a peak at 2 h and then decreased with time in all of the experimental groups (0.05, 0.10, and 0.20 mol/L groups). The Mn\(^{2+}\) concentrations in the aqueous humor decreased to almost normal levels in the epithelium-intact subgroups of 0.05 mol/L and 0.10 mol/L at 24 h [Table 1]. The peak concentration of Mn\(^{2+}\) in the vitreous humor occurred at 12 h and then decreased with time in all of the experimental groups (0.05, 0.10, and 0.20 mol/L groups). The concentration level of Mn\(^{2+}\) in the vitreous humor showed no significant change in the epithelium-intact subgroup of 0.05 mol/L [Table 2].

In the aqueous humor, the peak concentrations of Mn\(^{2+}\) in the epithelium-removed subgroup were much higher than that in the epithelium-intact subgroup. The peak concentration of Mn\(^{2+}\) in the aqueous humor increased with the increased concentration gradient of Mn\(^{2+}\) in all of the experimental groups. Corresponding to 0.05 mol/L, 0.10 mol/L, and 0.20 mol/L, the peak concentrations in the aqueous humor were 1813 ± 437 ng/ml, 5087 ± 666 ng/ml, and 22920 ± 1188 ng/ml, respectively, in the epithelium-removed subgroup and 197 ± 26 ng/ml, 549 ± 64 ng/ml, and 4286 ± 897 ng/ml, respectively, in the epithelium-intact subgroup [Table 1].

In the vitreous humor, the peak Mn\(^{2+}\) concentrations in the epithelium-removed subgroup were also much higher than that in the epithelium-intact subgroup. The peak concentration of Mn\(^{2+}\) in the vitreous humor also increased with the increased concentration gradient of Mn\(^{2+}\) in all of the experimental groups. Corresponding to 0.05 mol/L, 0.10 mol/L, and 0.20 mol/L, the peak concentrations in the vitreous humor were 155 ± 55 ng/ml, 884 ± 78 ng/ml, and 2556 ± 492 ng/ml, respectively, in the epithelium-removed subgroup and 6.30 ± 2.32 ng/ml, 21 ± 6 ng/ml, and 63 ± 13 ng/ml, respectively, in the epithelium-intact subgroup [Table 2].

Manganese-enhanced magnetic resonance imaging of the visual pathway in the different groups
The 2D MIP T1 images demonstrated that the ON and SC of the visual pathway were enhanced in the epithelium-removed subgroups of 0.10 mol/L and 0.20 mol/L. The ON and SC were not effectively enhanced in the epithelium-removed subgroup of 0.05 mol/L and the epithelium-intact subgroup [Figure 2a]. The CNR of the ON and SC increased with the increased concentration gradient of Mn\(^{2+}\) in the epithelium-removed subgroups. The CNR was higher in the epithelium-removed subgroup than that in the epithelium-intact subgroup [Figure 2b].

There was a linear correlation between the CNR and Mn\(^{2+}\) concentrations in the vitreous humor. In the epithelium-removed subgroup, the Pearson’s correlation coefficient was 0.95 \((P < 0.05)\). In the epithelium-intact subgroup, the Pearson’s correlation coefficient was 0.56 \((P < 0.05)\).

Observation of Mn\(^{2+}\) corneal toxicity
One day after topical administration, corneal edema occurred in the epithelium-removed and epithelium-intact subgroups of 0.20 mol/L. Corneal transparency was not affected in the epithelium-intact subgroups of 0.05 mol/L and 0.10 mol/L. Fourteen days after Mn\(^{2+}\) administration, corneal edema still existed in the epithelium-removed and epithelium-intact subgroups of 0.20 mol/L. However, corneal edema was alleviated in the epithelium-removed subgroup of 0.05 mol/L. Corneal transparency remained normal in the epithelium-intact subgroups of 0.05 mol/L and 0.10 mol/L [Figure 3]. The corneal edema condition was analyzed with Fisher’s exact test, which revealed that 0.20 mol/L was significantly different from 0.05 mol/L and 0.10 mol/L in the epithelium-intact subgroup \((P < 0.05)\).

Fourteen days after topical administration, corneal HE staining showed that the corneal endothelium was severely damaged, and the corneal stroma was edematous in the epithelium-removed and epithelium-intact subgroups of 0.20 mol/L. However, the corneal endothelium and corneal stroma were morphologically normal in the epithelium-intact subgroups of 0.05 mol/L and 0.10 mol/L [Figure 4].

DISCUSSION
Our study showed that the corneal epithelium is a vital protective barrier that plays an important role in hindering Mn\(^{2+}\) permeating into the eye. There was a 10 h interval between the peak concentrations of Mn\(^{2+}\) in the aqueous and vitreous humors. An effective concentration of Mn\(^{2+}\) forming in the rabbit’s vitreous body was needed for MRI enhancement of the visual pathway. The epithelium-intact rabbit’s cornea could sustain the frequent administration of MnCl\(_2\) solution with a concentration lower than 0.10 mol/L.

In our study, we chose rabbits as the experimental model, which was different from mouse models used in prior
The corneal epithelium as a biological membrane barrier is one of the most important determinants of the pharmacokinetic processes of a drug or Mn\(^{2+}\).\[2,3,6,19\] In the present study, the epithelium-removed model fully illustrated the impeding role of the corneal epithelium in the topical administration of Mn\(^{2+}\). Our study revealed that the peak concentration of Mn\(^{2+}\) was directly affected by the epithelium and concentration gradient of Mn\(^{2+}\). A passive diffusion mechanism of Mn\(^{2+}\) was obvious because the concentration of Mn\(^{2+}\) in the aqueous and vitreous humors increased with the concentration gradients of the MnCl\(_2\) solution regardless of whether the cornea was epithelium-removed or epithelium-intact. Our results showed that destruction of the epithelial barrier resulted in a dramatic increase in the concentration of Mn\(^{2+}\) in the aqueous and vitreous humors. We concluded that the corneal epithelium played an important role in controlling the amount of Mn\(^{2+}\) permeating into the eye.

Our study also demonstrated that the intraocular concentrations of Mn\(^{2+}\) in rabbits fluctuated with time. The peak times of the aqueous and vitreous humors were 2 h and 12 h, respectively. This result was similar to a previous study that also revealed that the intraocular concentration of Mn\(^{2+}\) after topical administration fluctuated over time in a rabbit eye.\[17\] There was a 10-h lag in peak time between the aqueous and vitreous humors. We speculated that the iris and lens septum might be an intraocular physiological barrier for hindering Mn\(^{2+}\) from permeating into the vitreous body. It was noteworthy that the permeation of Mn\(^{2+}\) from the aqueous humor to the vitreous humor also complied with a passive diffusion mechanism. However, the precondition of this passive diffusion mechanism was that the concentration of Mn\(^{2+}\) in the aqueous humor should reach a relatively high level due to the existence of the iris and lens septum barrier. Our study showed that the elevation in concentrations of Mn\(^{2+}\) in the vitreous humor was not significant when the peak concentration of Mn\(^{2+}\) in the aqueous humor was lower than 197 ± 26 ng/ml.

Our prior study revealed that the CNR value increased with the injection concentration of Mn\(^{2+}\) until the injection

![Table 1: Concentration of Mn\(^{2+}\) in the aqueous humor (ng/ml), \(n = 5\)](image)

![Table 2: Concentration of Mn\(^{2+}\) in the vitreous humor (ng/ml), \(n = 5\)](image)

\[P \leq 0.05\] compared with control, \[P < 0.05\] compared between epithelium-removed subgroup and epithelium-intact subgroup.
concentration reached 75 mmol/L. This finding indicated that CNR was directly proportional to the concentration of Mn\(^{2+}\) in the vitreous humor within certain limits. In the present study, we also found a positive correlation between the CNR and the Mn\(^{2+}\) concentration of the vitreous humor. The visual pathway was significantly enhanced in the epithelium-removed subgroups of 0.10 mol/L and 0.20 mol/L, of which concentrations of Mn\(^{2+}\) in the vitreous humor were 884 ± 78 ng/ml and 2556 ± 492 ng/ml, respectively. This result suggested that an effective concentration of Mn\(^{2+}\) in the vitreous humor was required because the retinal cells must have enough free Mn\(^{2+}\) to allow absorption to form an ideal MEMRI image. However, our result was different from the result of the prior study in which the topically loaded Mn\(^{2+}\) did not diffuse into the vitreous space but rather might have been absorbed into the iris to diffuse or travel.
Because the prior study did not measure the change in concentration of Mn$^{2+}$ in the vitreous humor after topical administration, these two contradictory results might be ascribed to the diversity of animal models or the MRI pulse sequences with a low sensitivity that are needed to develop and further refine sensitivity to small changes during relaxation times.

The cornea is a vulnerable refractive tissue in the ocular refractive system. The transparency of the cornea is vital for crisp vision. Transparency of the cornea is maintained by an endothelium that is vulnerable to toxins. Excessive Mn$^{2+}$ can induce corneal toxicity. A previous study revealed that anterior chamber injections of a MnCl$_2$ solution resulted in corneal endothelium degeneration. High concentrations of Mn$^{2+}$ (0.75 mol/L and 1.00 mol/L) can cause corneal damage via topical administration. Although we reduced the concentration of Mn$^{2+}$ to a relatively low level (0.05, 0.10, and 0.20 mol/L), Mn$^{2+}$ corneal toxicity still cannot be disregarded. Endothelium degeneration was inevitable after losing the protective epithelium. Our study demonstrated that repeated administration of Mn$^{2+}$ at 0.20 mol/L, rather than at 0.05 mol/L and 0.10 mol/L, in the epithelium-intact eyes also caused severe edema. This finding indicated that concentrations of Mn$^{2+}$ above 0.20 mol/L might destroy the epithelium barrier and that the cornea with an intact epithelium could sustain the concentration of Mn$^{2+}$ under 0.10 mol/L. We speculate that the combined action of the Mn$^{2+}$ toxicity itself and hypertonic dehydrated environment might be responsible for the breakdown of the epithelium barrier and the endothelium.

Our research investigated the permeation of Mn$^{2+}$ in the aqueous and vitreous humors, evaluated the MRI enhancement of the visual pathway and observed the corneal toxicity of Mn$^{2+}$. Some study limitations still exist in our research. For example, the concentrations of Mn$^{2+}$ in the ON and SC were not measured; thus, we could not determine the relationship between the CNR and the concentration of Mn$^{2+}$ accurately in the ON and SC. In addition, we observed the corneal toxicity only with the HE staining technique, and the subcellular structural damage was not studied; therefore, latent long-term corneal toxicity is not well understood.

To effectively and safely achieve MEMRI of the visual pathway via topical administration, the following research experiments still need to be performed: (1) synthesizing a small-molecule compound of Mn$^{2+}$ that meets the requirements, such as being nontoxic, highly permeable to the cornea, and transportable between neurons; (2) improving MRI pulse sequences to make them more sensitive to small changes in relaxation times and Mn$^{2+}$ concentrations; and (3) ascertaining the relationship between the effective concentration of Mn$^{2+}$ in the vitreous humor and MRI enhancement effect. Once those obstacles are overcome, MEMRI via topical administration will become an excellent tool for modern neuroscience studies.

In conclusion, the topical administration of Mn$^{2+}$ is not an available drug delivery route for MEMRI of the visual pathway at present. The corneal epithelium is a barrier to Mn$^{2+}$, and the iris and lens septum might be another intraocular barrier to the permeation of Mn$^{2+}$. An elevated Mn$^{2+}$ concentration contributed to the increased permeation of Mn$^{2+}$, higher MEMRI signals, and corneal toxicity. The MRI enhancement of the visual pathway requires an effective Mn$^{2+}$ concentration in the vitreous body.

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Conflicts of interest
There are no conflicts of interest.

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