Abstract. The aim of the present study was to investigate the protective effects of miconazole on myelin sheaths following cerebral white matter damage (WMD) in premature infant rats. Sprague Dawley rats (3-days-old) were randomly divided into four groups (n=30 each) as follows: Sham surgery group, WMD model group, 10 mg/kg/day treatment group and 40 mg/kg/day treatment group. A cerebral white matter lesion model was created by ligating the right common carotid artery for 80 min. Treatment groups were administered with 10 or 40 mg/kg miconazole at 4-8 days following birth (early treatment group) or 5-11 days following birth (late treatment group). Rats in the model group received the same concentration of dimethylsulfoxide. Myelin basic protein (MBP) immunohistochemical staining and western blotting were used to detect the expression of cerebral white matter-specific MBP, and changes in myelin structure were observed using transmission electron microscopy. No swelling or necrosis was observed in the corpus callosum of the sham group rats, whereas rats in the model group demonstrated edema, loose structure, fiber disorder, inflammatory gliocytes and selective white matter lesions. Following treatment with miconazole, MBP expression in the corpus callosum was significantly higher compared with the model group. Furthermore, in the model group, myelin sheaths in the corpus callosum were loose with small vacuoles, there was a marked decrease in thickness and structural damage was observed. Conversely, a marked improvement in myelination was observed in the treatment group. The results of the present study suggest that miconazole is able to promote formation of the myelin sheath to ameliorate premature cerebral white matter lesions caused by ischemia or hypoxia in rats.

Introduction

Oligodendrocytes (OLs) are major cells of the central nervous system (CNS) and are critical for neuronal function (1). CNS myelin abnormalities are features of several neurological disorders (2), including white matter injury (3), multiple sclerosis (4) and neuromyelitis optica (5). The causes of demyelination typically include immunological injury, excitotoxicity, viral infection, dystrophia and oxidative stress (6). At present, there are two methods used to treat demyelinating diseases; the first is reducing demyelination and the second is promoting remyelination (7). The therapeutic approaches include transplanting OL progenitor cells (OPCs) to protect endogenous OLs, or increasing the activity of exogenous OPCs to reduce demyelination and promote remyelination (8). Several studies have reported that the survival rate of transplanted OPCs is low, that few OPCs differentiate into mature OLs and that transplanting exogenous OPCs can lead to host rejection (9). Therefore, the aim of the present study was to explore a more effective method of promoting myelination by protecting and increasing the activity of endogenous OPCs.

Stimulating an endogenous regenerative response may be a potential effective treatment for chronic demyelinating diseases, including multiple sclerosis. Recently, Najm et al (10) reported that miconazole promotes OPC differentiation and induces remyelination in demyelinating disease models via mitogen-activated protein kinase (MEK)-dependent activation of extracellular signal-regulated kinase (ERK)1/2. Their study investigated the miconazole-promoted remyelination in a lysolecithin-induced demyelinating adult mouse model. In the present study, whether miconazole also serves a therapeutic role in premature infant cerebral white matter lesions was investigated.

White matter damage (WMD) is the most common type of cerebral lesion and cause of neural-developmental disorders, including premature cognitive defects and cerebral palsy, in premature infants (11). The pathogenesis of WMD typically includes changes in cerebral blood flow and cerebral tissue metabolism, which leads to a loss of OPCs and mature OLs,
causing cerebral white matter lesions (12). Ischemic hypoxia and infection are the main causes of cerebral white matter lesions in premature infants (13). At present, there is no effective treatment for premature infants with hypoxia and ischemic cerebral lesions. The aim of the present study was to explore the effect of miconazole on myelin regeneration and formation in the corpus callosum of rats with WMD.

Materials and methods

Experimental animals. Sprague-Dawley dams with litter were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). A total of 120 3-day-old Sprague Dawley rat pups (62 males and 58 females, mean weight, 7-8 g) from 12 litters were housed in a clean environment at 15-28°C with 45-44% humidity and a 12 h light/dark cycle, and provided with adequate food and water. All experimental procedures were approved by the Animal Ethical and Welfare Committee of the Navy General Hospital of People’s Liberation Army (Beijing, China).

Grouping and drug delivery. Experimental animals were randomly divided into four groups (n=30/group): The sham surgery, model, 10 mg/kg miconazole treatment and 40 mg/kg miconazole treatment groups. The treatment groups were administered with intraperitoneal injections of 10 or 40 mg/kg/day miconazole (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) at 4-8 days of age (the early treatment group consisting of 10 rats from each group) or 5-11 days of age (the late treatment group consisting of 10 rats from each group). The model group was administered with intraperitoneal injections of dimethylsulfoxide (DMSO) at the same concentration, whereas rats in the sham group received no treatment.

Establishment of the WMD model. At 3 days old, the rats were anesthetized using sodium pentobarbital (Shanghai Xinya Pharmaceutical, Co., Ltd., Shanghai, China) in a sterile environment. The limbs were fixed in the supine position on the operating table, the skin was sterilized with iodine, and a midline incision was made at the neck to reveal the right common carotid artery, internal jugular vein and vagus nerve. The right common carotid artery was ligated and the skin incision was subsequently sutured (14). In the sham group, only the right common carotid artery was exposed. Rats were placed on a heating mat at 37°C following the surgery for recovery, and subsequently housed in an environment containing 6% oxygen and 94% nitrogen at 33°C to provide constant anoxia for 80 min before being returned to their mother’s cage.

Brain tissue sections. At either days 8 or days 12 post-surgery, rats (n=3 per group) were anesthetized by intraperitoneal injection with pentobarbital sodium (50 mg/kg), the thoracic cavity was opened and the right auricle was dissected. A total of ~100 ml saline followed by ~100 ml paraformaldehyde were perfused into vascular system via the left ventricle. The rats were then decapitated, and brain tissue was harvested and fixed in 4% paraformaldehyde for 24 h at 4°C. Tissues were subsequently dehydrated in 30% sucrose solution until the tissue blocks sank to the bottom. Serial sections of the decanted brain tissue (10-µm-thick) were then cut.

Hematoxylin and eosin (H&E) staining. Brain tissue were fixed in 4% paraformaldehyde solution at room temperature for 24 h, embedded in paraffin and cut into continuous sections (5-µm-thick) starting from the anterior fontanel. Sections were dehydrated in a graded series of alcohol, washed in distilled water and stained with hematoxylin for 3 min at room temperature. Sections were subsequently mounted and observed under an optical microscope at a magnification of x200.

Myelin basic protein (MBP) immunohistochemical staining. Brain tissue sections were washed in PBS, digested in 0.03% Triton X-100 and blocked with 5% sheep serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at 18-24°C. Tissues were incubated in anti-MBP antibodies (cat. no. ab62631; Abcam, Cambridge, UK; 1:200) at 4°C overnight, washed three times with PBS and then incubated with goat anti mouse secondary antibodies (1:500; cat. no. 115-165-068; Jackson ImmunoResearch Europe Ltd., Newmarket, UK) for 2 h at room temperature in the dark. The sections were washed again three times with PBS, observed under a fluorescence microscope and images were captured at a magnification of x200. ImagePro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to convert the fluorescent images into black and white images, and the integrated optical density (IOD) value was measured.

Western blotting. At either days 8 or days 12 post-surgery, protein was extracted from the corpus callosum of rats (n=3 per group) using a Minute™ Total Protein Extraction kit (cat no. SD-001/SN-002; Invent Biotechnologies, Co., Ltd, Beijing, China) according to the manufacturer's protocols and quantified as previously described (10). Samples were denatured and equal amounts of protein (30 µg/lane) were loaded, separated by 10% SDS-PAGE and transferred to a PVDF membrane overnight. The membrane was blocked with 5% skim milk for 30 min at room temperature, incubated with anti-MBP antibodies (1:1,000) for 12 h at 4°C and subsequently incubated with horseradish peroxidase-conjugated goat anti mouse secondary antibodies for 30 min at room temperature. Protein bands were developed using enhanced chemiluminescence (cat no. 29050; Engreen Co., Ltd, Beijing, China). ImageJ software 2.1.4.7 (National Institutes of Health, Bethesda, MA, USA) was used to calculate the expression of MBP relative to GAPDH (cat no. GB12002; Wuhan Servicebio Biotechnology Co., Ltd, Wuhan, China) via densitometry. The dilution of the anti-GAPDH antibody was 1:1,000.

Transmission electron microscopy. At either days 8 or days 12 post-surgery, sections (1-3 mm thick) from the right corpus callosum of rats (n=3 per group) were fixed in 2% glutaraldehyde at 4°C for 72 h and then in 1% osmic acid at 4°C for 2 h. The sections were dehydrated using a series of 30-100% acetone and embedded in epoxy resin embedding medium. Myelin cross-sectional slices (50-nm-thick) were obtained using an ultramicrotome. Sections were observed under a transmission electron microscope and images were
captured for further analysis. A total of 800 root axonal fibers were counted at a magnification of x15,000. Following image acquisition, axon and myelin diameters were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) and the G-ratio value (axon diameter/myelin diameter) was calculated as previously described (15).

**Neuroethology tests.** The following neuroethology tests were performed in a blinded manner at postnatal day 28.

**Tail suspension test.** A tail suspension test was performed as previously reported (16), with minor modifications. Rat forelimbs were allowed to grasp a glass rod (diameter, 0.5 cm) at a height of 45 cm, and the time taken between being placed on the rod and falling off was recorded and scored. Scoring was as follows: <10 sec, 1 point; >10-30 sec, 2 points; >30-120 sec, 3 points; >120-300 sec, 4 points; >300 sec, 5 points.

**Slope test.** Rats were placed with their heads at a 45° decline and the time taken for rats to turn their heads up >135° was recorded (17).

**Field test.** The bottom of a 30x30x30 cm square box was divided into nine equal squares with chalk lines. Rats were placed in the central square and their activity was observed for 30 min. Scoring was performed as follows: 1 point for each instance where >50% of the rat's body moved to an adjacent square and 1 point whenever the rat was observed standing on hind legs.

**Statistical analysis.** Data are presented as the mean ± standard deviation and statistical analysis was performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). One-way ANOVA followed by a post-hoc Tukey's test was used to examine differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Successful establishment of the WMD model.** At 4 days post-surgery, H&E staining revealed that the corpus callosum structure in rats from the sham group was normal; cells had a dense and uniform distribution, the brain tissue outline was clear, and no cell swelling, necrosis or pathological changes were observed (Fig. 1A and B). Conversely, swelling, necrosis, nucleus pycnosis, loose structure, fiber disorder, enlarged lateral ventricles, hyperplastic inflammatory gliocytes, and active and selective white matter lesions were observed in the model group (Fig. 1C and D). MBP immunohistochemical staining results demonstrated that the corpus callosum in the sham group had no distinct myelin loss (Fig. 2A and B); however, significant myelin loss was observed in the white matter outside the sac on the surgical side of the model group compared with the non-surgical side and the sham group (P<0.05; Fig. 2C-E). These results indicate that the WMD model was successfully established.

**Miconazole treatment increases MBP expression in the corpus callosum of rats with WMD.** MBP immunohistochemical staining results revealed significant myelin loss on the surgical side of the corpus callosum in the model control group (P<0.05; Fig. 2E). Rats treated with 10 or 40 mg/kg/day miconazole in
the early (Fig. 3) and late (Fig. 4) treatment groups demonstrated a significantly higher expression of MBP compared with the model group (P<0.05). Furthermore, the IOD of rats in the 40 mg/kg/day treatment group was significantly higher compared with the rats treated with 10 mg/kg/day miconazole in the early and late treatment groups (P<0.05).

**Miconazole treatment increases MBP expression and enhances ERK1/2 activity in the corpus callosum of rats with WMD.** Western blotting results also revealed that the protein expression of MBP was increased in the miconazole treatment groups compared with the model group (P<0.05); however, no significant differences were observed between the 40 and 10 mg/kg/day groups (Figs. 5 and 6). These results indicate that miconazole is able to alter the protein expression of MBP in the corpus callosum, which suggests that it exerts a protective role during hypoxic ischemia. Furthermore, miconazole significantly upregulated MBP expression and enhanced ERK1/2 activity, as revealed by increased phosphorylated-ERK expression compared with the model group.

**Miconazole treatment improves the morphological features of WMD.** Transmission electron microscopy results revealed...
Table I. Neuroethology test results.

| Group          | Suspension test | Slope test (sec) | Field test |
|----------------|-----------------|------------------|------------|
| Sham operation | 3.6±0.97a-c     | 3.2±0.92a-c      | 13.4±1.43a-c |
| Model          | 1.5±0.53b-c     | 9.6±1.5b-c       | 5.9±1.73b-c |
| 10 mg/kg miconazole | 2.4±0.84c     | 6.6±1.1c         | 7.3±1.34c   |
| 40 mg/kg miconazole | 2.7±0.67      | 6.9±1.6          | 7.5±1.35    |
| P-value        | <0.05d          | <0.05e           | <0.05f      |

*P<0.05 vs. model group, *P<0.05 vs. 10 mg/kg group, *P<0.05 vs. 40 mg/kg group, *F value=12.617, *F value=40.419, and *F value=51.154.

that, at either day 8 or day 12 post-surgery, little myelination was present in the model group, nerve fibers were irregularly shaped and arranged loosely, and myelin thickness was markedly decreased (Fig. 7). Following miconazole treatment, the quantity of myelin was markedly increased compared with the control group, indicating that demyelination was ameliorated and myelin thickness was improved. G-ratio analysis demonstrated that the G-ratio in the control group was significantly higher compared with the miconazole treatment groups, while the 40 mg/kg miconazole treatment group G-ratio value was lower compared with the 10 mg/kg treatment group (data not shown). These results suggest that miconazole is able to improve cerebral white matter injury in premature infants.

**Miconazole treatment improves the neurological function of rats with WMD.** Neuroethology tests were performed to assess the effect of miconazole treatment on the functionality of rats with WMD (Table 1). In the tail suspension test, rats in the miconazole treatment groups demonstrated flexibility, agility and accuracy of movement, and were able to turn and crawl along the glass rod with a long suspension time. Compared with the treatment groups, rats in the model group were unresponsive with decreased autokinetic movement, flexibility and stability. Furthermore, they exhibited shaky lower limbs and were unable to stay on the rod for extended periods. Rats in the sham group scored significantly higher in the suspension test compared with the model and treatment groups (P<0.05). In the slope test, rats in the sham group took a significantly shorter amount of time to move compared with the model and treatment groups (P<0.05); however, the times recorded for the treatment groups were significantly shorter compared with the model group (P<0.05). Lastly, in the field test the sham group scored significantly higher compared with the model and treatment groups (P<0.05), whereas the model group scored significantly lower compared with the treatment groups (P<0.05) (Table I). No significant differences were observed between the 10 and 40 mg/kg/day miconazole treatment groups for any of the neuroethology tests.

**Discussion**

Cerebral white matter lesions in premature infants may cause long periods of movement and cognitive dysfunction, which is one of the main causes of cerebral palsy and mental retardation in children (11). Infants with very low birth weights (<1,500 g) have a high risk of premature infant cerebral white matter lesions, with 10-15% developing movement dysfunction, and 25-50% presenting with cognitive and behavioral deficits or social dysfunction (18). This is the primary cause of nervous system developmental disorders in premature infants and negatively affects survival (18). At present, there is no effective treatment for WMD (19). Cerebral OPC injury is the main cause of WMD, resulting in myelin destruction and delayed remyelination (20). In recent years, researchers have performed exogenous OPC transplants in animal models in order to verify whether the OPCs are able to survive, differentiate into mature OLs and promote myelination (21). Webber et al (21) transplanted OPCs into portal vein ligation model rats and reported that, 7 weeks later, the majority of transplanted cells had survived and expressed OPC-specific markers, including neural/glial antigen 2, O-antigen 4 and OL-transcription factor 2, indicating that the transplanted OPCs did not differentiate into mature OLs. Another study transplanted OPCs induced from human neural stem cells into rat demyelination models; at 8 weeks the majority of cells were alive, but <2% expressed MBP, a specific marker of mature OLs (9). These results indicate that, although transplanted OPCs are able to migrate to the injury and survive for an extended period, the majority do not differentiate into mature OLs. This is similar to previous observations of WMD; when demyelinating lesions occur, endogenous OPCs migrate to the lesion site, but few differentiate into mature OLs (22). It has been reported that the microenvironment of the CNS is an important factor for neuron and axonal regeneration (23); as such, efforts have been made to improve the microenvironment when attempting to identify effective targets for the promotion of axonal regeneration (24).

The aim of the present study was to promote endogenous OPC survival, differentiation and myelin formation in order to treat WMD. Muscarine receptor antagonists, including benztrapine and clemastine, have previously been reported to improve multiple sclerosis and promote myelin regeneration (10). The results of the present study demonstrated that miconazole,
another member of the family that has been approved by the US Food and Drug Administration as a fungal infection treat-
ment (10), is also able to promote OPC differentiation in vitro. Miconazole is also able to promote lyssolecithin-induced
demyelinating rat endogenous OPC differentiation, where the number of mature OLs is 13x that of the controls and axon
myelination is almost 12x (10). This indicates that miconazole serves an important regulatory role in the early stages of OPC
differentiation. In the present study, it was investigated whether miconazole was also able to promote endogenous OPC dif-
ferration in a rat model of infant WMD and whether it induced myelin formation following ischemia or hypoxia.

The CNS of 7-day-old rats is the same as that of term or near-term neonates, which has formed early myelin (25).
Unilateral carotid artery ligation combined with hypoxia induces brain lesions in the unilateral cortex and hippocampal neurons,
whereas it does not produce significant white matter lesions. Rat cerebral white matter is vulnerable 2-3 days after birth,
at which point it consists mainly of OPCs (26). As such, immature 3-day-old rats are ideal for studying premature cerebral white
matter injury (26). In the present study, H&E staining and MBP immunohistochemical staining was used to verify that the WMD
model was successfully established following carotid artery ligation. H&E staining revealed swollen cells, dilated intercel-
lular spaces, a disordered cellular arrangement, tiny infarcts and white matter injuries of the corpus callosum in the model group,
and MBP immunohistochemical staining revealed significant demyelination. These results indicate that the animal model of
hypoxic ischemic cerebral white matter injury was successful.

The protective effect and underlying mechanisms of miconazole on the myelin sheath in 3-day-old rats with cerebral white
matter injury were also investigated. The experimental results provide a basis for the potential clinical use of miconazole as
a treatment for WMD. MBP immunohistochemical staining and western blotting results revealed that MBP expression
was higher in the corpus callosum of miconazole-treated rats compared with the model controls, and that treatment with
40 mg/kg was more effective compared with 10 mg/kg. The results of transmission electron microscopy further indicated that
treatment with miconazole ameliorates ischemia/anoxia-induced WMD via promoting myelination in a rat model of ischemic
brain injury. This protection is implemented via the activation of ERK phosphorylation. The protective effect of miconazole
in the rat brain makes it an ideal candidate for further investigation as a clinical treatment for WMD.

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