Ganglioside GM1 reduces white matter damage in neonatal rats

Xiao Rong, Wei Zhou*, Xiao-Wen Chen, Li Tao, and Juan Tang

Department of Neonatology, Guangzhou Women and Children’s Medical Center, Guangzhou Medical College, Guangzhou, China, *Email: zhouwei_pu02@126.com

This study investigated the neuronal protective effect of monosialotetrahexosylganglioside (GM1) on the hypoxia-ischemia white matter damage (WMD) in neonatal rats. Brain hypoxia-ischemia was induced by bilateral carotid artery occlusion in 4-day-old neonatal rats. Bilateral carotid artery occlusion (BCAO) was performed in rats in WMD and GM1 groups, while in sham group; the rat bilateral carotid arteries were merely exposed without occlusion. Immunohistochemical staining was used to determine the expression of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and β-amyloid precursor protein (β-APP). In addition, suspension test, slope test, and open-field test were carried out on day 26 after BCAO to determine the neurobehavioral function. The percentage of MBP-positive cells was decreased while β-APP-and GFAP-positive cells were increased in WMD group. After treated with GM1, the percentage of MBP-positive cells increased significantly than WMD rats at post-operation 72 h and day 7. GFAP-positive cells and β-APP-positive cells decreased significantly in WMD group at post-operation 72 h, day 7 and 26. The suspension test, slope test, and open-field test showed that neurobehavioral function was improved in ganglioside GM1 group compared with WMD group. Taken together, our findings suggested that ganglioside GM1 treatment reduces hypoxia-ischemia induced impairment of the neurobehavioral function in WMD in neonatal rats.

Key words: white matter damage, ganglioside GM1, neurobehavioral function

INTRODUCTION

White matter damage (WMD) is the predominant form of brain damage in premature infants and may lead to serious complications such as cerebral palsy, cognitive and visual-audio dysfunction. Up to date, no ideally effective treatment for symptoms in WMD was developed. The direct pathogenesis factors for WMD include ischemia and hypoxia, which impairs cerebrovascular autoregulatory function. It has been shown that susceptibility of the late oligodendrocyte precursors to ischemia and hypoxia is the etiology of WMD in premature children (Khwaja and Volpe 2008). Therefore, it is an urgent need to prevent and treat brain injury such as WMD in premature infants in a timely effective manner. This study sought to provide a neuronal mechanism underlying an effective treatment for WMD.

Previous studies have shown that the single sialic acid hexose ganglioside GM1 is the only ganglioside that passes through the blood–brain barrier. GM1 can integrate into the membranes of neurons and plays a nutritious role in maintaining physiological function of neurons. Furthermore, GM1 repairs central nervous system damage during cerebral ischemia or hypoxia and trauma (Saito et al. 1999, Jiang and Gu 2003).
Although GM1 is widely used in the treatment of neonatal hypoxic-ischemic encephalopathy, the neuronal mechanisms involved in the neuroprotective effect in the treatment of WMD are not clear (Jin et al. 2006, Tanaka et al. 2010, Ong et al. 2012, Yang et al. 2012).

In this study, we used GM1 to treat neonatal rats with WMD in the early stage and determined the alterations of the microstructure and molecular biomarkers, as well as the neurobehavioral function during GM1 treatment. The findings from the current study provide a basis and a rational for the clinical application of GM1 to treat WMD in premature infants.

METHODS

WMD rat model

Neonatal Sprague-Dawley (SD) rats (either sex, 10–14 g) were used and provided by the Experimental Animal Center of Guangdong Province. These rats were randomly divided into sham-operated, WMD, and GM1 (WMD rats treated with GM1) groups. The WMD rat model was prepared as described previously (Uehara et al. 1999). Briefly, 4-day-old SD rats were anesthetized by ether and put in a supine position. Bilateral common carotid arteries were separated and were ligated by suture. In sham-operated rats, the common carotid arteries were only exposed without ligation. The wound was closed with suture and the rats were put in a 37°C incubator to allow their recovery from the anesthesia. WMD rats were treated with saline (0.01 ml/g, i.p.) while the rats in GM1 group were treated with GM1 (0.02 mg in 0.01 ml saline/g, i.p.) for 5 times in intervals of 24 h. After each injection, the rats were returned to their home cage and stayed with their mommy rats.

Hematoxylin and Eosin (HE) staining and immunohistochemical staining

The rats were deeply anesthetized with 20% urethane at post-operation 24 h, 48 h, 72 h, day 7 and day 26. These rats were intracardiacally perfused with heparin-containing saline followed by 4% paraformaldehyde. The brain was removed, fixed, and prepared for paraffin sections. For HE staining, consecutive coronal slides were sectioned caudal from Bregma at thickness of 5 μm. These sections included lateral ventricles, striatum, corpus callosum and internal capsule. Conventional HE staining was used to evaluate WMD including leukoaraiosis, cavity formation, coagulation necrosis, and lateral ventricles dilatation. For immunohistochemical staining: paraffin sections were made

![Fig. 1. GM1 treatment reduced neuronal injury. HE staining showed GM1 reduced the morphological alterations in periventricular region in sham, WMD, GM1 rats at post-operation 24 h, 48 h, 72 h, day 7 and day 26. Note that GM1 treatment reduced the neuronal edema, apoptosis, and patchy necrosis at post-operation 24 h, 48 h, and 72 h compared with WMD rats (A). Also, GM1 treatment reduced the loosened tissue structure and enlargement of cerebral ventricles in WMD rats (B). (Post-OP) post-operation. Scale bars are 50 μm in A and 0.5 mm in B. Each group had 10 rats and the given panels were representatives of each group, respectively.](image)
through anterior fontanelle point and the midline dorsal hippocampal level. Myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), and β-amyloid precursor protein (β-APP) were immunohistochemically stained. Briefly, sections were incubated with primary antibodies for MBP (dilution: 1:100), GFAP (dilution: 1:150) and β-APP (dilution 1:150), respectively. Then, sections were reacted with diaminobenzidine tetrahydrochloride. The stained cells were identified under an inverted microscope (Olympus, Japan). Cells with positive staining were counted under a light microscope in high magnificent view (400×). Cell numbers were calculated as the mean of 10 visual fields randomly selected in one specimen.

Suspension test: the rat forepaws were forced to grab a horizontal glass rod (diameter 0.5 cm), which was 45 cm high above the table surface. Then, we recorded the time until the rats released its forepaw and fell. The grabbing score was calculated as: 1: less than 10 s; 2: between 10 and 30 s; 3: between 31 and 60 s; 4: between 61 and 300 s; 5: more than 300 s. Slope test: The rat was placed in an upside-down position on an inclined plane with an angle at 45° to the desk level. We recorded the time for rats turning its head upward with an angle more than 135°. Open-field test: Animals were removed from its home cage and placed individually into a box [36 cm (L) × 36 cm (W) × 36 cm (H)]. The bottom of the box was equally divided into nine squares with black ink. The rats were placed in the central square for 30 s. We recorded one score in the case rats moved half of its body to cross the border of the central square and enter adjacent squares or the rats stranded with its hind legs. The total scores for each rat were summarized and compared between groups.

GM1 were obtained from the TRB Pharma SA production, Argentina. MBP, GFAP, and beta-APP antibodies were purchased from Wuhan Boster Biological Engineering Co., Ltd., China. The HRP sheep antimouse rabbit IgG polymer were obtained from Fujian new bio-technology development Co., Ltd., China. Data were expressed as means ± SEM. SPSS (version15.0) was used for statistical analysis. For comparisons of difference between datasets, the repeated-measures analysis of variance (ANOVA) with Dunnett’s test was performed to compare responses within or between experimental groups. \( P<0.05 \) was considered statistically significant.

RESULTS

General observation

Rats in WMD and GM1 groups displayed varying degrees of abnormal symptoms including pale skin, cyanosis, poor circulation, and frequent convulsion-like nodding in 30 min after surgery and were improved after 1~2 h. At post-operation 72 h, all rats in WMD and GM1 groups displayed obvious depression myotonic convulsions and tremors (4 rats in WMD group) and limb tremor without obvious convulsions (3 rats in GM1 group). Rats in WMD and GM1 groups displayed significant low body weight, unresponsive, reduced spontaneous motor activity compared with sham-operated rats.

GM1 treatment reduced neuronal injury in WMD rats

Under light microscope, neurons in periventricular white matter in sham-operated rats displayed an even distribution. The bilateral cerebral ventricles were symmetry in size. However, in WMD (n=10) and GM1 group (n=10) of rats, neurons in periventricular regions displayed edema in soma and dendrites at post-operation 24 h. At post-operation 48 h, neurons in periventricular regions showed apoptosis-like changes such as

![Fig. 2. GM1 treatment reduced neurochemical alterations in WMD rats. Immunocytochemistry staining showed MBP, β-APP, and GFAP-positive neurons in periventricular region in sham, WMD, GM1 rats at post-operation day 7. Each group had 10 rats and the given panels were representatives of each group, respectively. (WMD) white matter damage.](image-url)
cytoplasmic enrichment, nuclear chromatin condensation, apoptotic bodies in blue-black color, puncture necrosis. At post-operation 72 h, the neurons in this region revealed derangement, edema, enlarged gap between cells, sieve-like white matter necrosis, lysed nucleus fragmentation, and patchy necrosis (Fig. 1A). At post-operation day 7, the periventricular white matter and internal capsule displayed a lighter and loosened staining compared with that in sham-operated rats. Also, tissue slides displayed cavities in the internal capsule and enlargement of bilateral cerebral ventricles; The lateral cerebral ventricles were significantly enlarged at post-operation 26 day (Fig. 2B). Compared with WMD rats, the range of neuronal necrosis was smaller in GM1-treated rats at post-operation 72 h (Fig. 1A). Furthermore, the lateral ventricles enlargement was smaller than in GM1-treated rats than in WMD rats at post-operation day 7 and 26. These data suggest that GM1 treatment reduces the structural impairment in WMD rats.

**GM1 treatment reduced neurochemical alterations in WMD rats**

The MBP immuno-positive neurons were evenly distributed around lateral ventricles. The number of MBP-positive neurons was significantly lower in WMD rats ($n=10$, postoperative 24 h, 48 h, 72 h, day 7) and GM1 rats than in sham-operated rats. MBP-positive neurons in GM1 rats did not differ from WMD rats at postoperative 24 h, 48 h ($P>0.05$), but were significantly higher at post-operation 72 h and day 7 than in WMD rats ($P<0.01$). Figures 2 and 3 showed MBP immunohistochemical staining positive cells in sham-operated, WMD, and GM1 rats at each time point. The percentage of β-APP-positive neurons was significantly increased in WMD rats compared with sham-operated rats at each post-operation time points except postoperative day 26. However, compared with sham-operated rats, the percentage of β-APP-positive neurons in GM1 rats had no significant differences at post-operation 72 h, day 7 and 26. Furthermore, the percentage of β-APP-positive cells in GM1 rats did not differ significantly from WMD rats at post-operation 24 h and 48 h, but was significantly decreased at post-operation 72 h, day 7 and 26 ($n=10$, $P<0.01$, Fig. 3). Figure 2 showed β-APP immunohistochemical staining in sham-operated, WMD, and GM1 rats.

In addition, we examined the neuronal GFAP expression at each time points. The percentage of GFAP-positive neurons in WMD rats was significantly higher than in sham-operated rats ($n=10$, $P<0.05$, Figs 2 and 3). Although GFAP-positive cells in GM1 rats at 24 h and 48 h was not significant different from WMD rats, the percentage of GFAP-positive cells in GM1 rats were significantly lower than WMD rats at 72 h, day 7 and 26 ($n=10$, $P<0.01$, Fig. 3). These data indicated that GM1 treatment suppresses the neurochemical alterations in WMD rats.

**GM1 treatment attenuated the neurobehavioral dysfunction in WMD rats**

We determined effect of GM1 treatment on neurobehavioral changes in WMD rats by using suspen-

Fig. 3. Effect of GM1 on the MBP, β-APP, and GFAP-positive neurons in sham, WMD, and GM1 rats. Summary data showing the percentage of MBP, β-APP, and GFAP-positive neurons in periventricular region in sham ($n=10$), WMD ($n=10$), GM1 rats ($n=10$). Data were expressed as mean ± SEM, *$P<0.05$ compared with sham. #*$P<0.05$ compared with WMD rats.
sion test, slope test, and open-field test. In suspension and open-field tests, the scores of WMD rats were significantly lower than that in sham-operated rats ($n=10$, $P<0.05$, Fig. 4). Furthermore, in slope test, the time for rat to turn around its head was significantly longer in WMD rats than that in sham-operated rats. GM1 treatment significantly increased the scores in suspension and open-field tests (Fig. 4A and B), although the scores of these two tests in GM1 rats were still lower than sham-operated rats. Furthermore, the turning around time in slope test was significantly short in GM1-treated rats than in WMD rats. These data suggest that GM1 treatment reduces the neurobehavioral impairment in WMD rats.

**DISCUSSION**

Establishing a suitable animal model is one of the key factors to study mechanisms of WMD. Because ischemia and hypoxia are critical in the pathogenesis of WMD, which including impairment of cerebrovascular and oligodendrocytes precursors of glial cells function (Sha and Zhou 2008), we used an ischemia-hypoxia rat model in this study. WMD is characterized by deep white matter injury, focal necrosis, and damage of oligodendrocytes precursor cells (Volpe 2001). The pathology of WMD includes reduced white matter volume, ventricular dilatation, and lack of myelin (Mallard et al. 2003). It has been shown that WMD displays damage of oligodendrocyte precursor cells, decrease in myelin formation, proliferation of astrocytes, and axonal injury (Blumenthal 2004). Consistently, the HE staining of the brain tissue revealed that neural injuries including cell edema and gap between cells enlargement in the periventricular white matter, oligodendrocyte apoptosis and necrosis. Furthermore, cortical and periventricular white matter expressed a loose and liquid stove structure, cysts formation and enlarged ventricles. In addition, neurobehavioral function tests revealed that the WMD rats were unresponsive and the spontaneous motor activity and flexibility reduced. Also, these rats displayed a reduced stability of the suspension. In the slopes and open-field tests, the scores in WMD rats were lower than sham-operated rats, suggesting the neurobehavioral function is impaired in WMD rats. These symptoms observed in this experimental model of rat WMD were consistent with the clinical symptoms in premature infant patient with WMD.

GM1 belongs to gangliosides family and plays an important role in interaction between cells and matrix proteins and is critical in the regulation of cell membrane function (Avrova et al. 1998). GM1 has been widely used to treat neonatal hypoxic-ischemic encephalopathy, Parkinson’s disease, acute cerebral infarction, peripheral neuropathy, retinal ischemia and spinal cord injury and other diseases. GM1 exerts its action through the following aspects (Tyurin et al. 1992, Ferrari and Greene 1996, Avrova et al. 1998): (1) exogenous GM1 can cross the blood-brain barrier to protect cell membranes through a rescue of Na⁺–K⁺ ATPase and Ca²⁺–Mg²⁺ ATPase activity to maintain a balance of ions and reduce water accumulation within the neurons; (2) GM1 can inhibit NO production during hypoxia, inhibit excessive activation of glutamate

![Fig. 4](image-url). GM1 treatment reduced the neurobehavioral impairment in WMD rats. Summary data showing that GM1 treatment reduced neurobehaviors in suspension test (A, $n=10$), slope test (B, $n=10$), and open-field test (C, $n=10$). Data were expressed as mean ± SEM, *$P<0.05$ compared with sham. #$P<0.05$ compared with WMD rats.
receptors, against the neurotoxicity of excitatory amino acids and free radicals, enhance the activity of antioxidant enzymes, reduce lipid peroxidation, and eliminate damage to the cell membrane by free radical, reduce the release of excitatory amino acids; (3) increase blood flow to damaged brain tissue, and promote axonal growth and improve the survival of neurons; (4) regulate synaptic signaling, improve nerve conduction velocity, and promote the recovery of the brain electrical waves, thereby reducing secondary brain injury neurotoxicity; (5) enhance neurotrophic activity to strengthen neural remodeling, promote recovery after brain injury; (6) promote the expression of Bcl-2, inhibit the expression of Bax, then inhibit cell withered death through increasing TrkA phosphorylation, and (7) promote the effect of neurotropic factor, promote neuronal development and repair the damage of the neurons (Rabin et al. 2002, Marconi et al. 2005).

MBP is a marker protein for the oligodendrocyte and the quantitative analysis of MBP can be used as a sensitive indicator for the detection of oligodendrocyte injury. Because MBP expression is limited in nervous system, it is a specific biochemical indicator for WMD, especially the myelin sheath damage (Wang 2010). We found that the number of MBP-positive cells were reduced in WMD rats at post-operation 24 h, 48 h, 72 h, and day 7, suggesting that ischemia and hypoxia lead to oligodendrocyte precursor cell injury. Importantly, we found that GM1 treatment significantly reduced this damage as evidenced by a significant increase in MBP-positive cells. It is most likely that GM1 promotes the repair of oligodendrocyte myelination in WMD. The number of MBP-positive cells was significantly reduced in rats at post-operation day 30. This reduction of MBP-positive cells is probably due to the maturation of oligodendrocyte.

The developing axon is vulnerable to the injuries induced by ischemia and hypoxia in WMD animals (Haynes et al. 2005). The pathological basis of cerebral palsy is lacking continuity and myelination in the axons. The β-APP is a marker protein for axonal damage and is transported via microtubule in cytoskeleton. The β-APP plays primary role in the regulation of synapses formation (Priller et al. 2006) and neural plasticity (Turner et al. 2003). It has been shown that β-APP is one of the essential components for the delivery of the enzymatic Aβ production (Zheng and Koo 2006). β-APP mediates the axonal transport of β-secretase and presenilin-1, which are principal processing enzymes that generate amyloid-β from β-APP (De Strooper et al. 1998, Vassar et al. 1999). The process occurs in an axonal membrane compartment transported by kinesin-I (Kamal et al. 2001). Because ischemia-hypoxia injury may result in impairment of axonal transportation, the collapse of the cytoskeleton and increase in β-APP transcription lead to β-APP aggregation (Tohda et al. 2004). Also, cerebral ischemia and hypoxia can lead to the β-APP abnormal split, increase of amyloid formation and serious cellular injury (Boyt et al. 2000). In this study, β-APP-positive cells in WMD rats increased after carotid artery ligation at different time points, indicating the presence of axonal injury. GM1 treatment significantly decreased β-APP-positive cell number at postoperative 72 h, day 7, and day 26. These data suggest that GM1 promotes axonal recovery after damage.

GFAP is an intermediate filament protein in soma and dendrites of astrocyte. GFAP expression level represents the injury levels of astrocyte in white matter. Proliferation of astrocytes is a common response to trauma or inflammation in the central nervous system and is characterized by increased number and hypertrophy of astrocyte, and increased expression of GFAP. Previous studies have shown that the occurrence of WMD is closely related to an increase in GFAP-positive astrocyte number (Mallard et al. 2003, Pang et al. 2003). We found that GFAP-positive astrocyte increased with an enlargement of cell size in WMD rats, suggesting that the astrocytes were activated. GM1 treatment significantly decreased GFAP expression at different time points. These data suggest that GM1 is able to inhibit the proliferation of astrocytes.

WMD can result in motor, cognitive, and behavioral disorders. Neurological behavior disorders are stabilized in early childhood. It is accepted that 30-day old rats are equivalent of 2–5 year-old children. The neurobehavioral dysfunction in rats with WMD is also equivalent to the typical clinical symptoms in the stage of cerebral palsy in human (Li et al. 2004). Because of the clinical significance, we selected 30-day old rats for neurological behavior test. Suspension test and slope tests mainly reflect the limb muscle strength and body balance. These tests are important to determine the voluntary body movement. Open-field test reflects the ability of emotional behavior. Thus, we used a combination of the above experiments to determine the neurobehavioral function. We found that long-term sensory, motor function and emotional capacity were
significantly lower in WMD rats compared with sham-operated rats. However, GM1 early intervention therapy in WMD rats significantly improved neurobehavioral function, suggesting that GM1 treatment significantly improves muscle strength, balance function, and the capacity of the voluntary movement.

CONCLUSION

Short-term follow-up case studies show that GM1 treatment improves children’s neurobehavioral function, reduces periventricular leukomalacia and persistent occurrence of lesions, and improves the prognosis (Zhang et al. 2008, Sun 2009, Li and Sun 2010). However, these studies are not entirely randomized controlled trials. Therefore, large-scale multi-center clinical trials are needed to further validate the neural protective effect of GM1 on cerebral WMD in premature infants.

ACKNOWLEDGEMENTS

This study was supported by The Science and Technology Project of Guangdong Province (Social Development): Early diagnosis and intervention study of white matter damage in preterm infants (2006B36030006). The authors have no conflicts of interest to report.

REFERENCES


