Temporal and spatial changes in oligodendrocytes in different brain regions following intrauterine ischemia in neonatal Wistar rats

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Hypoxia-ischemia (HI) is a common cause of fetal and neonatal white matter damage that is closely related to cerebral palsy, mental retardation, and other neurological disorders. Oligodendrocytes are an important white matter component. As myelin-forming glial cells in the CNS, they surround the axons of nerve fibers to form the myelin sheath, which plays an important role in mediating the rapid electrical conduction of axons. Human fetuses of 23-32 week gestational age, corresponding to day 2-5 postnatal rats,[1] have the greatest risk of developing periventricular leukomalacia following HI. At this stage, the subcortical white matter oligodendrocytes consist primarily of late oligodendrocyte progenitors, which are early cells involved in myelin formation. Current research on the effect of HI on the developing brain has focused largely on 2-3 day-old rats, which correspond to premature human infants,[2] or 7 day-old rats, which correspond to near full-term human infants.[3] A study of the overall changes to oligodendrocytes in rats born within 28 days after being exposed to varying degrees of intrauterine HI may outline the changing patterns of oligodendrocytes post-HI in premature and full-term brains. The objective of the present study was to examine the spatial and temporal patterns of oligodendrocytes in neonatal Wistar rats following intrauterine ischemia, by tracking the number of myelin basic protein (MBP)-positive oligodendrocytes and MBP protein expression over a 28-day time course.

This study was performed in the Department of Pathology, Bethune Medical College of Jilin University, Changchun, China between February 2009 and January 2010. Ethical approval was received prior to conducting this study. Ninety-day-old female Wistar rats, weighing 280 ± 20 g, were provided by Gaoxin Experimental Research Center of Medical Animal, Changchun, China. A rat model of varying degrees of acute intrauterine distress was constructed by ligating the bilateral uterine arteries of pregnant rats.[4] Day 0 gestation was defined as the day when a vaginal plug was found. Full-term pregnant rats (21 days) were subjected to cervical dislocation, and secured to a dissection board. After disinfecting the abdomen, the abdominal cavity was cut open along the median ventral line, the uterus was fully exposed and isolated, and the bilateral uterine horns and uterine arteries were clamped with non-invasive hemostatic forceps to completely block the uterine blood supply. When the predetermined ischemia time (0, 15, or 25 minutes) was reached, the uterine wall was cut open, and fetal rats were extracted. Artificial respiratory stimulation was applied immediately to establish spontaneous breathing by the newborn rats. The above procedures were performed at ambient temperatures between 30-32°C. According to the duration of ischemia, 60 pregnant rats were divided into 3 groups: control group, whose uterus was surgically exposed, but whose uterine arteries were not occluded; mild HI group, and severe HI group, whose vessels of bilateral uterine horns and uterine body were completely clamped for 15 and 25 minutes. The newborn rats were breastfed by other female rats who delivered normally the day before. Ten newborn rats from each group (control, mild HI, and severe HI) were selected randomly at 0.5, 3, 6, and 24 hours, 3, 5, 7, 9, 11, 14, 21, and 28 days following brain ischemia-reperfusion, and were sacrificed using cervical dislocation. The skull was immediately stripped away, and all the brain tissues were removed and fixed in 10% neutral buffered formalin for 7 days. The brain was cut coronally into 2 sections at the middle part of mammillary bodies, conventionally dehydrated, and embedded in paraffin. The cortex, striatum, and hippocampus in each specimen were selected for analysis. Five serial sections were obtained, with a slice thickness of 4 um. Oligodendrocytes were labeled using rabbit anti-MBP immunohistochemistry (Fuzhou Maxim Biotech Inc., Fuzhou, China.). Under a 20X object lens, 6 non-overlapping visual fields were selected for each slice. Cells with brown cell membranes were regarded as positive cells. The positive cells were scanned with a MICROPHOT-FXA camera (Nikon, Tokyo, Japan), and stored in an image analysis system. Oligodendrocyte abundance was expressed according to the average number of positively stained cells within the reference area of each image obtained from 6 visual fields of each section. All data are expressed as mean ± standard deviation (SD). Statistical analyses were performed using the SAS 6.12 statistical software package (SAS Inc., Cary, NC, USA), and comparisons

Disclosure. The authors declare no conflicting interests, and this study was not supported or funded by any drug company.

between groups were conducted using ANOVA tests and the LSD test, a significant difference was assumed at $p<0.05$.

In the control group (**Figures 1A-1C**), MBP-positive cells were undetectable at 0.5, 3, 6, 24 hours, and 3, 5, or 7 days postnatally, but began to appear at day 9. The cells were small and spherical with few or no protrusions, and were located adjacent to the myelin sheath. The number of MBP-positive cells, and the level of MBP expression gradually increased over time in the control group. In the mild HI group (**Figures 1D-1F**), MBP-positive cells were absent at 0.5, 3, 6, 24 hours, and 3 days postnatally, but started to appear at 5 days postnatally. In the striatum, MBP-positive cells in the mild HI group gradually increased over time and were significantly higher than that of the control group at each time point. The number of MBP-positive cells in the severe HI group was higher than those in the control group, and was not significantly different from those of the mild HI group at postnatal days 5, 7, 9, and 14. However, the number of MBP-positive cells in the severe HI group was significantly lower than those in the control and mild HI groups at postnatal days 21 and 28 (**Table 1**). In the cortex, the number of MBP-

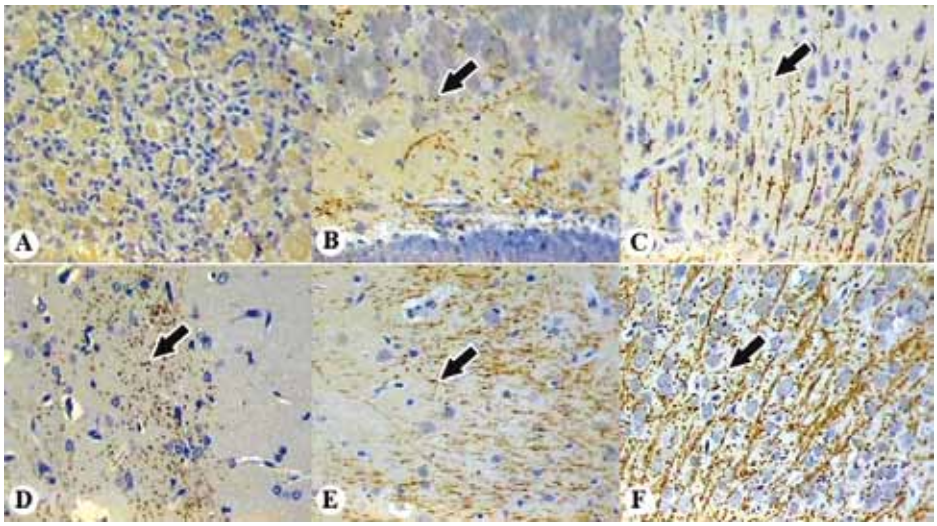

Figure 1 - Immunohistochemical images of changes in myelin basic protein (MBP)-positive cells at selected time points showing: **A)** The MBP-positive oligodendrocytes in the control group in the striatum at day 5 (200X): no MBP-positive cells were noted. **B)** The MBP-positive oligodendrocytes in the control group in the hippocampus at day 14 (200X): a few spherical MBP-positive cells appeared and a low level of myelination was observed. **C)** The MBP-positive oligodendrocytes in the control group in the cortex at day 28: a moderate number of spherical MBP-positive cells appeared, which were located adjacent to the myelin sheath. **D)** The MBP-positive oligodendrocytes in the mild hypoxia-ischemia (HI) group in the striatum at day 5: a few spherical MBP-positive cells were found and no myelination was identified. **E)** The MBP-positive oligodendrocytes in the mild HI group in the hippocampus at day 14: a moderate number of spherical MBP-positive cells and a low level of myelination were observed. **F)** The MBP-positive oligodendrocytes in the mild HI group in the cortex at day 28: a large number of spherical MBP-positive cells adjacent to the myelin sheath and considerable expression of the myelin sheath were noted.

Table 1 - Changes in the number of myelin basic protein (MBP)-positive cells in the striatum over time (5-28 days) in neonatal Wistar rats following intrauterine ischemia.

Postnatal age	Number	Control group	Mild HI group	Severe HI group	F	*P*-value
5 days	10	0	41.5±10.7*	49.9±11.2*	6.34	<0.001
7 days	10	0	79.5±25.1*	97.9±26.2*	8.67	<0.001
9 days	10	31.5±10.6	144.7±21.5*	156.7±29.6*	9.37	<0.001
14 days	10	76.4±26.6	221.7±25.9*	207.6±27.1*	10.34	<0.001
21 days	10	412.1±27.1	602.4±13.4*	183.6±15.7*†	13.76	<0.001
28 days	10	394.3±23.2	591.6±37.1*	196.1±17.7*†	12.05	<0.001

*compared with control group $p<0.001$, †compared with mild HI group $p<0.001$, HI - hypoxia-ischemia

positive cells in the mild HI group was significantly higher than that of the control group at postnatal days 5, 7, 9, and 28. The number of MBP-positive cells in the severe HI group was significantly increased relative to the control and mild HI groups at postnatal days 9, 14, 21, and 28 (**Table 2**). In the hippocampus, the number of MBP-positive cells in the mild HI group was significantly higher than that in the control group at each time point, there were no differences between the severe HI group and the mild HI group at postnatal days 5, 7, 9, and 14. The number of MBP-positive cells in the severe HI group was significantly lower than those in the control and mild HI groups at postnatal days 21 and 28 (**Table 3**).

The results of the current study showed that MBP-positive cells started to appear in the control group at day 9 after birth, while they appeared in the HI groups at day 5 after birth. Oligodendrocytes in neonatal rats within one week after birth are immature and cannot form myelin. Undifferentiated and immature oligodendrocytes are more prone to ischemic stress than differentiated and mature oligodendrocytes. The earlier appearance of MBP-positive cells in the HI groups is believed to be related to the release of neurotrophic factors and neurotransmitters by nerve cells and other glial cells following HI. These molecules can stimulate considerable levels of oligodendrocyte progenitor cell proliferation and maturation. Our results indicate mild HI may stimulate the proliferation of late oligodendrocyte progenitors, consistent with previous research.[5] However, our results also suggest that severe HI will reduce the number of mature oligodendrocytes, decreasing MBP expression and resulting in demyelination damage. The present study also noted that the number of MBP-positive cells varied in different brain regions. Unlike the striatum and the hippocampus, the number of MBP-positive cells in the cortex in the severe HI group was significantly higher than that in the control and mild HI groups. This observation further indicates that the response to HI varies in different brain regions, and that demyelinating oligodendrocytes may not necessarily die. Severe ischemic damage can permanently inhibit the expression of myelin-related genes, and even if the cells eventually survive, myelin synthesis will gradually decrease.

Table 2 - Changes in the number of myelin basic protein (MBP)-positive cells in the cortex over time (5-28 days) in neonatal Wistar rats following intrauterine ischemia.

Postnatal age	Number	Control group	Mild HI group	Severe HI group	F	*P*-value
5 days	10	0	51.9±17.1*	67.1±18.2*	7.61	<0.001
7 days	10	0	94.2±23.5*	120.4±24.1*	8.16	<0.001
9 days	10	49.7±11.9	105.9±20.6*	197.1±20.7*†	10.71	<0.001
14 days	10	191.7±26.7	170.6±27.9*	256.7±28.4*†	9.24	<0.001
21 days	10	306.6±27.9	317.7±26.1*	386.9±34.5*†	8.76	<0.001
28 days	10	416.7±36.1	632.6±27.2*	739.9±37.6*†	12.34	<0.001

*compared with control group $p<0.05$, †compared with mild HI group $p<0.05$, HI - hypoxia-ischemia

Table 3 - Changes in the number of myelin basic protein (MBP)-positive cells in the hippocampus over time (5-28 days) in neonatal Wistar rats following intrauterine ischemia.

Postnatal age	Number	Control group	Mild HI group	Severe HI group	F	*P*-value
5 days	10	0	45.1±20.1*	50.7±20.5*	7.17	<0.001
7 days	10	0	51.7±29.1*	57.9±9.2*	8.91	<0.001
9 days	10	25.1±8.1	93.5±10.1*	97.7±11.2*	8.35	<0.001
14 days	10	30.3±7.6	146.7±19.6*	162.2±23.5*	9.17	<0.001
21 days	10	316.6±29.4	474.7±21.7*	241.4±23.5*†	13.79	<0.001
28 days	10	331.1±37.6	496.6±27.1*	212.6±34.6*†	14.17	<0.001

*compared with control group $p<0.001$, †compared with mild HI group $p<0.001$, HI - hypoxia-ischemia

Our study has some limitations. The phenomenon of the variation of oligodendrocytes in different spatial and temporal patterns were observed. Further studies on the mechanism of this phenomenon are needed, especially the relationship between oligodendrocyte death and demyelination should be further clarified.

In conclusion, HI can stimulate the differentiation and maturation of oligodendrocytes in a time- and area-dependent manner. In the same brain region, mild HI may stimulate hyperplasia of late oligodendrocytes, while severe HI may reduce the number of late oligodendrocytes and MBP expression.

Acknowledgment. *We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.*

Received 22nd January 2012. Accepted 2nd September 2012.

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