



Review

## Animal Models of Periventricular Leukomalacia

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Periventricular leukomalacia, specifically characterized as white matter injury, in neonates is strongly associated with the damage of pre-myelinating oligodendrocytes. Clinical data suggest that hypoxia-ischemia during delivery and intrauterine or neonatal infection-inflammation are important factors in the etiology of periventricular leukomalacia including cerebral palsy, a serious case exhibiting neurobehavioral deficits of periventricular leukomalacia. In order to explore the pathophysiological mechanisms of white matter injury and to better understand how infectious agents may affect the vulnerability of the immature brain to injury, novel animal models have been developed using hypoperfusion, microbes or bacterial products (lipopolysaccharide) and excitotoxins. Such efforts have developed rat models that produce predominantly white matter lesions by adopting combined hypoxia-ischemia technique on postnatal days 1-7, in which unilateral or bilateral carotid arteries of animals are occluded (ischemia) followed by 1-2 hour exposure to 6-8% oxygen environment (hypoxia). Furthermore, low doses of lipopolysaccharide that by themselves have no adverse-effects in 7-day-old rats, dramatically increase brain injury to hypoxic-ischemic challenge, implying that inflammation sensitizes the immature central nervous system. Therefore, among numerous models of periventricular leukomalacia, combination of hypoxia-ischemia-lipopolysaccharide might be one of the most-acceptable rodent models to induce extensive white matter injury and ensuing neurobehavioral deficits for the evaluation of candidate therapeutics.

**Key words:** Periventricular leukomalacia, white matter injury, cerebral palsy, hypoperfusion (hypoxia-ischemia), inflammation (lipopolysaccharide), premyelinating oligodendrocytes

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Cerebral palsy (CP), one of the most-devastating neural diseases, results from asphyxia during delivery as well as intrauterine infection [1]. The disease is also called periventricular leukomalacia (PVL), hypoxia-ischemia encephalopathy (HIE), white matter injury/damage (WMI/WMD), and CP in serious cases exhibiting neurobehavioral symptoms. Although asphyxia during delivery is considered an important etiological factor in many cases with PVL, the etiology might be multi-factorial. Infections and inflammation, coagulopathy and genetic

background alone or in combination seem to be important [2]. Furthermore, it is well known that respiratory dysfunction is a predominant factor in pre-term infants in which a very-high incidence of CP is produced [3-6]. Motor, perceptual, visual, behavioral and/or cognitive disorders occur in the majority of cases with PVL [7-9]. For a better understanding of the underlying mechanisms of WMI, several animal models of PVL have been developed based on the hypoxia-ischemia (HI) surgery, infection or lipopolysaccharide (LPS) administration,

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or excitotoxin challenge. In this review, we summarized these models of PVL and analyzed the association between infection/inflammation and cerebral palsy found in human infants [10].

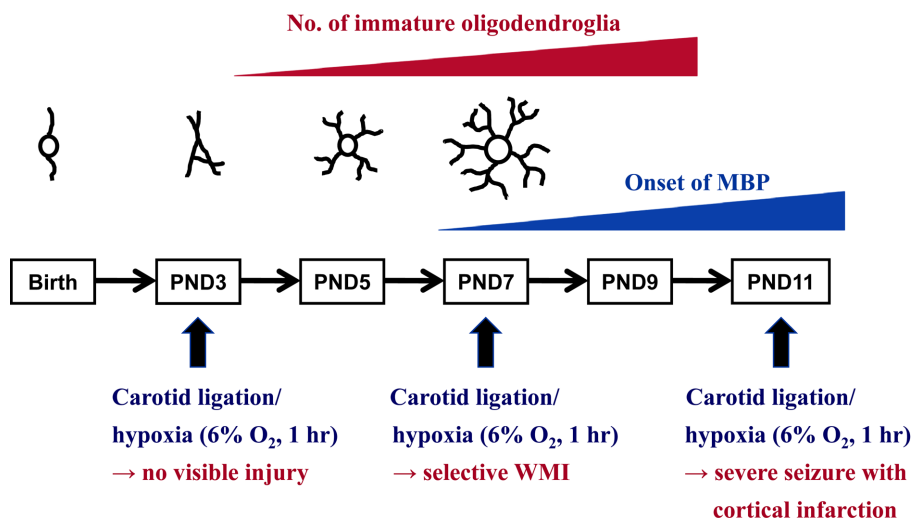
## Neuropathology of Infantile PVL

Although infantile WMI was named PVL [11], it seems like that WMI is widespread including periventricular, subcortical and callosal white matters, and internal capsule [12-15]. Injury of white matter usually predominates, however gray matter areas, such as the cerebral cortex, thalamus and basal ganglia, are also affected directly or secondarily due to loss of white matter according to the severity [14,16]. WMI lesions are shown to be focal or diffuse [17]. The focal lesions involve necrosis of all tissue constituents, neurons or axons, astrocytes and oligodendrocytes, which may lead to cavitory lesions with surrounding proliferating astrocytes (so called astrocytosis or hypertrophic astrocytes). In comparison, the diffuse type involves a broad paucity of white matter, thinning of the corpus callosum, and often ventriculomegaly in late stages [18]. Particularly, the diffuse injury is characterized by astrogliosis, loss of oligodendroglial lineage, and impaired myelinogenesis [19]. WMI usually occurs during delivery when the white matter is non-myelinated or in the initial phase of myelination, and populated mainly by late oligodendroglial progenitors ( $O4^+O1^-$ ), but with a small proportion of immature oligodendroglia ( $O4^+O1^+$ ) [20]. Axons may also be lost in the lesions as demonstrated by axonal retraction balls and clubs [11,21]. Accumulation of amyloid precursor proteins (APP) [22] and axonal transections [21] are seen in the focal lesions [23]. In addition to hypertrophic (activated) astrocytes, microglia and macrophages are also constituents of WMI

[14,15,22], and these cells are immunoreactive for the pro-inflammatory cytokines including tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) [24], suggestive of an inflammatory response. These cells may be reactivated residents of the white matter or recruited from the blood, indicating the possible participation of blood-derived inflammatory cells [25].

## Etiology of Infantile PVL

There are two major etiologies of PVL; i.e., ischemia (hypoxia)-reperfusion [26] and infection/inflammation, resulting in fetal inflammatory responses [27]. The ischemia-reperfusion process is confirmed by the presence of arterial end and border zones in the periventricular white matter [28,29], pressure-passive circulation without autoregulatory function [30], and the susceptibility of  $O4^+O1^-$  late oligodendroglial progenitors which are predominating oligodendroglial cells at gestational weeks 24-32 (GW24-32) or GW24-40 susceptible to oxidative stress, excitotoxicity and *in vitro* ischemia (Figure 1) [19,20,31-35]. On the other hand, inflammatory pathway is supported by the fact that WMI is predicted by histological chorioamnionitis and vasculitis in umbilical cord and chorion plate as well as pro-inflammatory cytokines such as IL-6 and IL-8 in amniotic fluid and fetal blood [27,36,37]. In addition, microglia/macrophages in white matter lesions exhibit immunoreactivity for IL-6 and TNF- $\alpha$  [24]. Premyelinating oligodendrocytes (pre-OLs), which have been shown to be a key cellular target in PVL, are in a phase of active development during GW24-40 [20,33-35]. Four developmental stages of oligodendroglial maturation include (1) oligodendroglial progenitors, (2) late oligodendroglial progenitors ( $O4^+O1^-$  pre-oligodendrocytes), (3) immature oligodendrocytes ( $O4^+O1^+$ ),



**Figure 1.** Developmental stage-dependent periventricular leukomalacia of rodents induced by hypoxia-ischemia. PND, post-natal day; WMI, white matter injury.

and (4) mature myelin-producing oligodendrocytes [myelin basic protein (MBP) positive (MBP<sup>+</sup>)]. Pre-oligodendrocytes (late oligodendroglial progenitors) and immature oligodendrocytes are referred as pre-OLs. These differentiating forms, especially the O4<sup>+</sup>O1<sup>+</sup> immature oligodendrocytes, ensheath axons for differentiation into myelin-producing oligodendrocytes. Mature, MBP-expressing and ultimately myelin-producing oligodendrocytes are not abundant in cerebral white matter until after term. During the peak period of PVL, O4<sup>+</sup>O1<sup>-</sup> late oligodendroglial progenitors predominate in cerebral white matter and at GW28 account for 90% of the total oligodendroglial population [20]. At GW28-40, O4<sup>+</sup>O1<sup>-</sup> cells begin differentiation into O4<sup>+</sup>O1<sup>+</sup> immature oligodendrocytes, which consists of approximately 30% of total oligodendrocyte population during the later premature period and about 50% by term. These two early differentiating cells are specifically vulnerable to injurious insults, such as ischemia and inflammation, which lead to excitotoxicity and generation of free radicals. These pre-OLs show enhanced vulnerability to the following factors: (1) reactive oxygen species (ROS) and reactive nitrogen species (RNS), because of impaired antioxidant defenses; (2) excitotoxicity due to exuberant expression of calcium-permeable glutamate receptors, and enhanced expression of the main glutamate transporter, which can become a source of injurious glutamate; and (3) cytokine injury, because of both expression of the interferon- $\gamma$  (IFN- $\gamma$ ) receptors on the pre-OLs in the context of pronounced availability of IFN- $\gamma$  in the abundant astrocytes of PVL, and sensitivity to injury by tumor-necrosis factor- $\alpha$  (TNF- $\alpha$ ), which is secreted by the abundant activated microglia [38-52].

Microglia play key roles during brain development, involving apoptosis, vascularization, axonal development, and myelination [53-56]. Thus, microglia become prominent in the forebrain at GW16-22 [57-60], reaching a peak in cerebral white matter in the third trimester [60]. It is believed that microglia be key effectors of cellular injury following ischemia and/or inflammation, since they generate ROS/RNS, secrete injurious cytokines, and enhance excitotoxicity [44-48,58,61-63]. Because microglia are abundant in normal cerebral white matter during peri-natal period, their activation leads to injury to white matter constituents including pre-OLs, and also axons and subplate neurons [60]. Not surprisingly, it was found out that many activated microglia are present diffusely in cerebral white matter in association with pre-OLs injury in PVL [39].

## Animal Models of PVL

According to clinical information described above, a large number of animal models of PVL have been developed. PVL

**Table 1.** Comparative time schedule for CNS development in animals and humans [1]

Animal	Human pre-term (GW23-36)	Human term (GW37-42)
Mouse	PND3-7	PND8-12
Rat	PND3-7	PND8-12
Rabbit	GD20-28 (70-85%)	Birth, GD33 (100%)
Dog	Birth	PNW>2
Sheep	GD93-99	GD119-133 (90%)

PND, post-natal day; GD, gestational day; PNW, post-natal week.

can be induced either by induction of a systemic inflammatory response through administration of bacteria or its products such as LPS [64-69], or ante- or post-natal HI surgery [68,70-73]. White matter lesions can also be induced by *N*-methyl-D-aspartate (NMDA) [65,74] and non-NMDA receptor [68] agonists, indicating that excitotoxicity may be involved in the development of PVL. The period prior to generalized myelinogenesis represents the developmental stage with a high vulnerability of white matter [19,65,73,75-78]. The white matter vulnerability has been related to the presence of O4<sup>+</sup>O1<sup>-</sup> pre-OLs at GW24-32 in humans [19,20,73]. The pre-OLs (O4<sup>+</sup>O1<sup>-</sup>) are susceptible to HI in immature rats, whereas O4<sup>+</sup>O1<sup>+</sup> immature oligodendrocytes are not [73]. Oligodendrocytes immunoreactive for O4 but negative for O1 predominate in the rat white matter on post-natal days 2-4 (PND2-4) [68,73], whereas O4<sup>+</sup>O1<sup>+</sup> cells predominate on PND7. This agrees with the suggestion in mice [65] that PND5 corresponds to GW24-30 in humans with regard to white matter maturation [77]. These studies suggest that white matter vulnerability in the rat/mouse on PND4, rather than PND7, would correspond to the white matter vulnerability in pre-term infants.

## Hypoperfusion models

In most of the HI models of PVL, both gray and white matters are affected, except for the dog model of ischemia [79] or hemorrhagic hypotension model [71] in fetal sheep, which produced selective WMI. In general, the pattern of WMI in rabbits, cats, dogs and sheep has a distribution and morphological features closer to human pre-term brain lesions (predominantly white matter) than that in rodents (Table 1) [1]. Induction of extensive WMI is difficult in rats and mice, which may be due to the different central nervous system (CNS) anatomy of rodents that have a much lower white/gray matter ratio. However, based on the cost efficiency, availability of antibodies and transgenic animals, rodent PVL models are acceptable. Earlier, a PVL model in immature animals with unilateral carotid ligation and exposure to 8% oxygen environment for 1-3 hours was introduced [80], in

which even though the vulnerability of myelinogenic zones was emphasized, most researches in this model have focused on gray matter injury. Nevertheless, it has been used successfully to study the response of immature oligodendroglial and stem/progenitor cells in white matter and periventricular zones to HI both in 9-day-old mice [81] and 7-day-old rats [73,82,83]. A corresponding model in 1-day-old rats has also been developed, which produces injury predominantly in the gray matter [84]. Recently, a modification model in rats, unilateral carotid artery ligation and 6% oxygen for 1 hour on PND7, was found to lead to selective loss of O1<sup>+</sup> oligodendroglia and decrease of MBP in the corpus callosum and periventricular white matter 4 days later, without affecting gray matter [68]. Another study induced a loss of O4<sup>+</sup> cells in the corpus callosum by bilateral carotid artery ligation and 10-min exposure to 8% oxygen environment [70]. In another study, permanent bilateral carotid artery ligation of PND5 or PND7 rats resulted in subcortical white and gray matter injuries [72,83]. However, because of limited survival beyond 2-3 days [83], 2-hour transient bilateral carotid ligation allowed long-term survival and still produced similar distribution of damage in subcortical regions. In comparison, permanent bilateral carotid artery ligation of 1-day-old rats allowed long-term survival longer than 2 weeks and produced WMI in corpus callosum, subcortex, internal capsule, and a significant enlargement of the ventricles [85] with limited pathology in the gray matter. Although rodent models need to be further explored, unilateral carotid artery ligation followed by exposure 8% oxygen for 2-3 hours is suitable, since various parameters to evaluate neurobehavioral deficits are obtainable, without mortality [83]. Interestingly, a model was introduced in rabbit fetuses of gestational days 21-25 (GD21-25), subjected to intrauterine hypoperfusion and WMI affecting O4<sup>+</sup>O1<sup>-</sup> pre-oligodendrocytes, were found 1-7 days after the insult [78]. Post-natal changes in magnetic resonance imaging (MRI) were found in the white matter and, moreover, severe neurological dysfunctions were demonstrated after birth approximately 10 days post-induction [86].

### Inflammation models

Extra-amniotic or intrauterine inoculation of live *E. coli* [67,87,88] without antibiotics induced pre-term stillbirth within 48 hours. In live pups sacrificed at 12-30 hours, brain injury was not detected [67,88]. However, antibiotic administration prevented pre-term delivery and fetal infection was delayed. Brain injury was detected in a part of pups after 2-6 days [87,88]. It is of interest to note that most fetuses with brain injury were blood culture negative, whereas there were inflammatory changes in chorioamniotic membranes. The

results indicate that brain injury is related to the sustained inflammatory response rather than to passage of microbes into the brain, which is supported by the lack of massive accumulation of granulocytes in the CNS lesions [67].

Underlying mechanisms of LPS-induced PVL are not fully known. LPS is believed to activate innate immune system via interaction with toll-like receptors (TLR) on immune cells [89]. LPS binds to CD14 that facilitates activation of TLR-4, which in turn, resulting in nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation and production of pro-inflammatory cytokines [89,90]. Thus, TLR-4- or CD14-deficient mice do not respond to LPS. TLR-4 receptors were found in the immature brain, and LPS administration induced an increased expression of CD14 [69]. In addition to the inflammatory response [91,92], LPS induces hypoperfusion [93], hypoglycemia [93], hyperthermia [91] and lactic acidosis, which may important factors triggering brain damage. In fact, a high dose of LPS (12 mg/kg) induced hypotension (50% decrease) as well as decrease in cerebral blood flow (CBF) in white matter, suggesting that hypoperfusion is an important contributor to injury in this region [93]. Interestingly, even a lower dose of LPS (4 mg/kg), leading to a 20-30% decrease in arterial blood pressure, was enough to induce widespread white matter lesions. Therefore, it is expected that appropriate (moderate) doses of LPS would induce hypoperfusion and injury of white matter. In young rabbits, intravenous injection of LPS (10 mg/kg) was found to decrease CBF by 25 and 43% in cerebral cortex and white matter, respectively [94]. However, brain injury was seen in the both regions, making it uncertain to what extent the decrease of CBF was the critical level for brain injury. Moreover, it was reported that WMI evolved in response to repeated doses of LPS in fetal sheep, in spite of the fact that hemodynamic effects became less pronounced, suggesting that hypoperfusion was not the critical factor [95]. Thus, it is suggested that the LPS-induced development of brain injury cannot be fully explained in terms of cerebral hypoperfusion. Especially, white or gray matter injuries in rats (PND5-7) were not successfully produced using various dose levels of LPS (0.3 mg/kg up to 100 mg/kg), in spite of a marked mRNA expression of pro-inflammatory cytokines in the white matter [66,69,96], implying that at least this form of CNS inflammation in rats was not sufficient to produce WMI. Such results indicate that LPS-induced WMI might be caused by a combination of systemic and CNS inflammatory effects.

### Excitotoxic models

Intracerebral administration of excitatory amino acid (EAA) receptor agonists [NMDA and DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxalone propionic acid (AMPA)] in PND7-14 rats

has been shown to induce lesions 40 times larger than in adult rats in the striatum, hippocampus and cerebral cortex [74]. Subcortical injection of ibotenate (an NMDA receptor agonist) in PND5 mice produced cortical and white matter injuries [65,97]. The topographical localization, ontogenetic window, and pharmacology of the lesions suggested that the lesions were primary, not secondary to the lesions in cerebral cortex or other gray matter areas. Indeed, microglia activation is triggered by NMDA, leading to extensive astroglial (but not oligodendroglial) death [97]. By comparison, injection of AMPA to PND7 rats caused selective WMI affecting O4<sup>+</sup>O1<sup>+</sup> pre-oligodendroglia (expressing GluR4 receptors), which was attenuated by NBQX (an AMPA receptor antagonist), but not by dizocilpine (MK-801; an NMDA receptor antagonist) [68]. Furthermore, in pre-oligodendroglial cultures, EAA-mediated inhibition of cystine uptake led to the depletion of glutathione and susceptibility to injury evoked by oxygen free radicals [31]. In spite of partial contradiction, these studies suggest that NMDA and AMPA receptors as well as non-receptor-mediated mechanisms may be involved in WMI. Injection of high doses of excitotoxins is highly artificial and the role of excitotoxicity in WMI remains obscure, but such paradigms may be useful to explain some aspects of human disease.

### Comparison of PVL models

In sheep fetuses, both LPS (100 ng) injection and asphyxia (25-min umbilical cord occlusion) caused periventricular (focal and diffuse) and subcortical (diffuse) WMI with a quite similar distribution, except for somewhat broader involvement of the gray matter (striatum and hippocampus) after umbilical cord occlusion. In both models, there were acute losses of glial fibrillary acidic protein (GFAP)-positive astroglia and cyclic nucleotide phosphohydrolase (CNPase)-positive immature oligodendroglia. In contrast, marked microglial responses were seen in both models, although microglial accumulation was more focal within the lesions induced by LPS. Furthermore, inflammatory cell infiltrations occurred much more frequently after LPS challenge, probably corresponding to macrophages/polymorphonuclear neutrophils (PMNs) found in other models of LPS-induced WMI in cats and dogs [91,92] or after local administration of LPS into the immature brain [98]. The results suggest that PVL evoked by a systemic inflammation (inflammatory WMI) has a different morphological appearance than that produced by hypoperfusion (ischemic WMI). And comparison of the underlying mechanisms of the two lesions may be of interest as these forms of injury may have clinical relevance [26,27]. The different distribution of microglia and macrophages following the two insults may be critical, because these cells appear to have a key role in ibotenate-induced

WMI [97], and because microglial toxicity depends on the density of microglia [99].

Only intraperitoneal administration of LPS to the pregnant rats or to the neonatal pups does not produce consistent brain injury [66,69]. However, LPS (0.3 mg/kg), intraperitoneally given 4 hours prior to HI in 7-day-old rats (brain maturity corresponding to near term), sensitized the brain to injury; 20-min HI plus LPS injection (HIL) induced extensive lesions in all animals, whereas 20-min HI alone produced essentially minimal or no injury [69]. In contrast, administration of another endotoxin, lipoteichoic acid, 3 hours prior to HI reduced brain injury [100]. Thus, further studies are necessary to find out whether this intriguing difference can be explained by the fact that LPS and lipoteichoic acid act via different receptors [90].

The potential of infections and inflammation in brain injury is not limited to pre-term infants. The relationship between infection/inflammation and PVL including CP may be higher in term rather than in pre-term infants [101,102]. Notably, chorioamnionitis was related to serious CP with 9-fold higher risk [10], in which the levels of pro-inflammatory cytokines and chemokines were much higher [103]. Moreover, the combination of intrauterine infection and asphyxia during delivery (hard labor) appeared to confer a synergistic effect with a substantially (78-fold) higher risk of CP [104], suggesting that inflammation (or infectious products) may sensitize the fetus to additional insults.

## Conclusion

Earlier, experimental researches on peri-natal brain injury have focused on hypoxic-ischemic damage to reflect the clinical problem of birth asphyxia, which has demonstrated critical clues on the pathophysiology of immature neuronal injury. However, only a small proportion of casualties suffering from PVL, especially CP, are related to asphyxia. In our recent understanding, intrauterine or peri-natal infection is emerged to be an important factor in pre-term as well as in term infants. Consequently, a number of novel animal models have been introduced in various species to demonstrate the features of WMI and explain underlying pathology in the early immature brain, either induced by hypoperfusion or infectious agents. Furthermore, recent studies have shown that bacterial endotoxin sensitize the brain to a secondary HI, supportive of the involvement of combinational factors in infantile brain injury. Conclusively, it is suggested that HIL models that consists of hypoxia-ischemia-LPS could be one of the best choices to induce extensive WMI and ensuing neurobehavioral deficits for the screening of candidate therapeutics.

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