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**Article** *in* Developmental Neuroscience · March 2005

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Dev Neurosci 2005;27:134–142
DOI: 10.1159/000085985

Received: August 30, 2004
Accepted: December 3, 2004

# Neuronal Injuries Induced by Perinatal Hypoxic-Ischemic Insults Are Potentiated by Prenatal Exposure to Lipopolysaccharide: Animal Model for Perinatally Acquired Encephalopathy



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**Key Words**
Inflammation · Hypoxia · Ischemia · Brain · Cerebral palsy

**Abstract**
We developed an original rat model for neonatal brain lesions whereby we explored the sequential effects of infectious and hypoxic-ischemic aggressions. We investigated the influence of combined exposure to *prenatal* infection with neonatal hypoxic-ischemic insult. Infectious effect was produced by administrating lipopolysaccharide (LPS) intraperitoneally to pregnant rats starting on embryonic day 17. Hypoxia-ischemia (H/I) was induced in the pups at postnatal day 1 (P1) by ligature of the right common carotid artery followed by exposure to hypoxia (8% $O_2$) for 3.5 h. Animals were randomized into four groups: (1) control group: pups born to mothers subjected to intraperitoneal saline injection; (2) LPS group: pups exposed in utero to LPS; (3) H/I group: pups exposed to postnatal hypoxia after ligation of the right carotid artery, and (4) H/I plus LPS group: in utero exposure to LPS followed by postnatal hypoxia after ligation of the right carotid artery. Neuropathological findings in pups examined at P3 and P8 showed that groups 2, 3, and 4 presented a pattern of neuronal injury similar to those characterized as 'selective neuronal necrosis' within the context of human perinatal encephalopathy. Neuronal cellular injuries were particularly seen in the neocortex, mainly in parasagittal areas. The extent of neuronal cell injury in the brain of rats exposed to postnatal H/I was significantly increased by antenatal exposure to LPS. This animal model provides an experimental means to explore the respective roles of anoxic and infectious components in the pathogenesis of perinatal brain lesions and consequent cerebral palsy.



## Introduction

Hypoxia/ischemia (H/I) is one of the pathophysiological processes leading to neonatal brain injury and subsequent lifelong disabilities affecting motricity, cognition and behavior [Freeman and Nelson, 1988; Nelson et al., 1998a, b; Volpe, 2001; Ferriero, 2004]. Mounting evidence from epidemiological, clinical and experimental studies shows that the innate immune response, particularly that involving proinflammatory cytokines and induced by prenatal infection, can also play a key role in perinatal brain damages [Bell and Hallenbeck, 2002; Bona et al., 1999; Cai et al., 2000; Damman et al., 2001;

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Duncan et al., 2002; Debillon et al., 2000; Grether and Nelson, 1997; Kadhim et al., 2001, 2002a, b; Kaukola et al., 2004; Mallard et al., 2003; Nelson et al., 1998a, 2003; Patrick and Smith, 2002; Peebles et al., 2003].

Brain injuries acquired during the neonatal period affect the white matter, especially in the premature newborn [Friede, 1989]. However, several studies [Inder et al., 1999; Volpe, 2001; Kinney and Armstrong, 2002; Kadhim et al., 2003], including recent investigations from our group, showed the presence of anomalies in the neonatal brain of both preterm and term patients affecting cerebrocortical neurons, such as foci of selective neuronal necrosis, reduction in the volume of cerebral cortical gray matter and hyperexpression of proinflammatory cytokines. So, perinatal aggressions involving H/I, often acting in combination with inflammation, seem to induce a continuum of neuropathologic damages in the immature brain, the so-called perinatally acquired encephalopathy, extending from white matter lesions in the preterm infant to parasagittal cerebral injuries in the term infant. On the other hand, selective neuronal necrosis seems to affect all gray matter regions of the brain and covers all gestational ages [Yager, 2004; Volpe, 2001].

Epidemiological studies showed that birth asphyxia, when associated with perinatal infection, results in an increase in the risk of developing spastic cerebral palsy [Nelson et al., 1998b]. Most of these perinatal infections begin in utero, whereas studies demonstrated the occurrence of cerebral palsy in association with antenatal chorioamnionitis or with increased concentrations of inflammatory markers in the amniotic fluid or cord blood [Baud et al., 1999; Bracci and Buonocore, 2003; Duggan et al., 2001; Murphy et al., 1995; Perlman et al., 1996; Wu et al., 2000, 2003; Yoon et al., 1996, 1997, 2003; Zupan et al., 1996]. The compounding effect of bacterial endotoxin in aggravating neonatal brain damage in H/I animals has already been reported in animal models for *postnatal* infection [Coumans et al., 2003; Eklind et al., 2001]. However, there has been as yet no model reproducing the precise order of events taking place in a large proportion of human newborns, namely *prenatal intrauterine* exposure to infection followed by later occurring asphyxia.

We therefore undertook this work: (1) to develop an animal model for perinatal brain lesions combining the effects of *prenatal* infection and early postnatal H/I, and (2) to study the impact of prenatal exposure to bacterial endotoxin on postnatal brain development, sequentially at postnatal day (P) 3 and P8, both in normal and deficient conditions of oxygen supply.

## Materials and Methods

*Animals*

Lewis pregnant rats were obtained from Charles River Laboratories (Saint-Constant, Queb., Canada) at embryonic day (E) 16. The rats were allowed to acclimate to our animal facility prior to experimental manipulations. They were kept at a constant temperature of 20°C, a 12-hour day/12-hour night rhythm, and were maintained on food and drink ad libitum. Animals were handled in accordance with the Animal Care and Use Committee at the University of Sherbrooke, Canada. Thirty-two gestational rats and a total of 81 born pups were included in this study. Clinical observations of the pups' behavior were performed daily from birth to P3 or P8. Motor behavior was observed twice a day, each time for 30–60 min. Each animal was observed for gross motor deficits with open-field locomotion, symmetry of turnover ability, and symmetry of muscle tone assessed by visual observation of resting animals. Feeding behavior was studied by checking twice a day the presence of milk in the pups' stomach. We also measured for each pup the increase in weight per 24 h from birth to P3 or P8. The pups were randomized to four experimental groups: (1) control group (group 1; n = 14): intraperitoneal saline injection to the pregnant rat; (2) lipopolysaccharide (LPS) group (group 2; n = 15): exposed in utero to LPS; (3) H/I group (group 3; n = 21): intraperitoneal saline injection to the pregnant rat followed by postnatal exposure to hypoxia after ligature of the right common carotid artery; (4) H/I plus LPS group (group 4; n = 31): in utero exposure to LPS combined with postnatal exposure to hypoxia after ligature of the right common carotid artery. In each experimental session, our four experimental conditions, including our control condition (group 1), were always simultaneously conducted.

*Experimental Design to Explore Effects of Prenatal LPS Administration on Perinatal H/I*

Pregnant rats (n = 20) were injected intraperitoneally with LPS (*Escherichia coli,* 0127:B8; Sigma, Oakville, Ont., Canada), or with saline solution (n = 12). These intraperitoneal injections were given every 12 h starting on E17 to the end of gestation. Pups were born at P0. Twenty-four hours after birth (P1), pups of both sexes and from different litters were exposed to H/I using slightly modified experimental methods originally described by Rice et al. [1981]. Thus, to induce ischemia, the right common carotid artery in these pups was exposed and ligated under isoflurane anesthesia (5% for induction, 2% for maintenance). The full duration of the surgical procedure was less than 5 min. Mortality rate associated with the surgical procedure was 1.6%. The pups were then returned to the dams. Thirty minutes later, these pups were exposed to 8% $O_2$/$NO_2$ balance, in a warm air incubator at ambient temperature of 36°C for 3.5 h. All animals survived hypoxia. Pups were left to recover for 15 min in an incubator at an air temperature of 36°C before being returned to the dams.

*Tissue Preparations and Histological Procedures*

At P3 or P8, after induced neonatal H/I, the rat pups were anesthetized by exposure to 5% isoflurane for 10 min and killed by decapitation. The rats were sacrificed at two different time points to study the evolution of the injury. We chose to sequentially study the evolution of the brain lesions at P3 and P8, which is in agreement with the histological data from a previous study about the timing of brain injury induced by H/I inflicted at P1 in newborn

rats [Sheldon et al., 1996]. The brain was removed and fixed in a buffered solution of 4% paraformaldehyde for 48 h. Each brain was cut coronally into three blocks for histological examinations. The plane of section was as follows: the 1st block was close to the bregma level (7.20 to 2.28 mm from the interaural line; –1.80 to –6.72 mm from the bregma); the 2nd one was situated anterior to the bregma (13.70 to 7.60 mm from the interaural line; 4.70 to – 1.40 mm from the bregma), and the 3rd one was posterior to the bregma level (2.20 to –2.60 mm from the interaural line; –6.80 to –11.60 mm from the bregma) according to classical stereotaxic coordinates [Paxinos and Watson, 1986]. Tissues were embedded in paraffin, sectioned at 5-μm coronal slices and stained with hematoxylin-eosin. We then determined the number and the extent of lesions by counting and measuring the cellular and architectural histopathological changes morphometrically using a Cool SNAP camera attached to an Olympus BX51 microscope and SigmaScan Pro hardware. The extent and the relative size of ensuing cerebral lesions were thus calculated by measuring the surface areas of histopathologically altered foci. We characterized areas of the brain as 'abnormal' clear-cut regions presenting an abundance of altered neural cells and nuclei, namely, hypereosinophilia, shrunken, dark or pyknotic nuclei, or blurring and dissolution of nuclear or cytoplasmic membranes on the basis of hematoxylin-eosin staining. The proportion of lesions was calculated by measuring the surfaces of affected areas; the percentages were obtained by dividing figures for the lesioned surface area by the total surface area of the neocortex in the ipsilateral hemisphere. In our experimental conditions, the relatively discrete brain lesions we observed (lack of massive damages/infarcts) did not alter the size of the hemisphere ipsilateral to the lesions enabling us to use the total surface area of the neocortex ipsilateral to the lesions as a reference in evaluating the extent of neuronal damages. In both H/I experimental groups (groups 3 and 4), hemispheres contralateral to carotid ligation were designated as 'hypoxic hemispheres'. To detect apoptotic cell death, adjacent sections to those used for hematoxylin-eosin staining were labeled using a TUNEL kit, according to the manufacturer's instructions (Roche, Laval, Que., Canada).

*Data Analysis*

Data were presented as means ± SEM. Comparisons between groups were performed using ANOVA, the Student-Newman-Keuls multiple comparison test, and t test with Welch correction. The significance level was set at $p < 0.05$.

## Results

### *Neuropathologic Alterations Induced by Prenatal Exposure to LPS (Group 2) as Compared to Prenatal Exposure to Saline (Group 1)*

LPS dose effect was first tested in 7 pregnant rats at different doses ranging from 200 to 800 μg/kg/12 h. The dose used in this study (200 μg/kg/12 h) is the dose that was associated with the lowest maternal, fetal and early neonatal mortality. Twenty-five pregnant rats were used in the following experiments. Our results are based on the study of the offspring from these 25 pregnant rats and from 2 out of the 7 pregnant rats we initially studied for LPS dose effect receiving 200 μg/kg/12 h. There was no significant difference in the mean duration of gestation (19.5 ± 0.4 days) in LPS (200 μg/kg/12 h)-exposed rats versus unexposed rats (19.1 ± 0.4 days). The mean number of pups in each dam was 6 ± 1.3 in dams exposed in utero to LPS versus 8.5 ± 1.3 in dams exposed in utero to saline ($p < 0.05$). No mortality was observed among the pregnant rats treated with LPS (200 μg/kg/12 h). A total of 81 pups were studied. Pups from LPS-treated pregnant rats had significantly lower body weights at P3 and P8 as compared to pups from saline-treated pregnant rats. The mean weight of pups in the LPS group (group 2) was 5.1 ± 0.2 g at P3, and 12.5 ± 0.8 g at P8, versus 8.4 ± 0.1 g at P3 and 15.9 ± 1.7 g at P8 in the control group (group 1) ($p < 0.01$). Exposure to LPS (group 2) induced a decrease in the brain weight at P8 as compared to control animals (group 1) ($p = 0.07$; table 1). No difference in motor or feeding behaviors was observed in pups exposed in utero to LPS as compared to pups exposed in utero to saline. Examination of the brain showed a normal macroscopic appearance at P3 and P8 in the newborn pups exposed in utero to LPS (group 2; n = 15) as well as in controls (group 1; n = 14). Microscopic examination of hematoxylin-eosin-stained tissue sections from LPS-exposed pups (group 2) revealed the presence of multiple foci of neuronal cytoarchitectural changes (fig. 1) presenting a similar aspect at P3 and P8. These lesions were de-

**Table 1.** Comparison of brain weights of the pups, at P3 and P8, in our different experimental conditions

| Age | Brain weight, g | | | |
|---|---|---|---|---|
| | control (group 1) | H/I (group 3) | LPS (group 2) | LPS + H/I (group 4) |
| P3 | 0.40 ± 0.00 (n = 3) | 0.44 ± 0.02 (n = 5) | 0.35 ± 0.02 (n= 2) | 0.36 ± 0.02 (n = 7) |
| P8 | 0.87 ± 0.04 (n = 6) | 0.93 ± 0.02 (n = 11) | 0.76 ± 0.03 (n = 9) | 0.78 ± 0.02 (n = 20) |

At P3, there was no significant difference between the mean brain weights of rat pups from our different experimental groups. At P8, the only significant difference ($p > 0.001$) was between groups 3 and 4, i.e. the mean brain weights of rat pups submitted to LPS + H/I (group 4) were decreased as compared with the mean brain weights of rat pups submitted to H/I alone (group 3). Exposure to LPS (group 2) alone induced a decrease in the brain weight as compared to control animals (group 1) ($p = 0.07$). Results are means ± SEM.

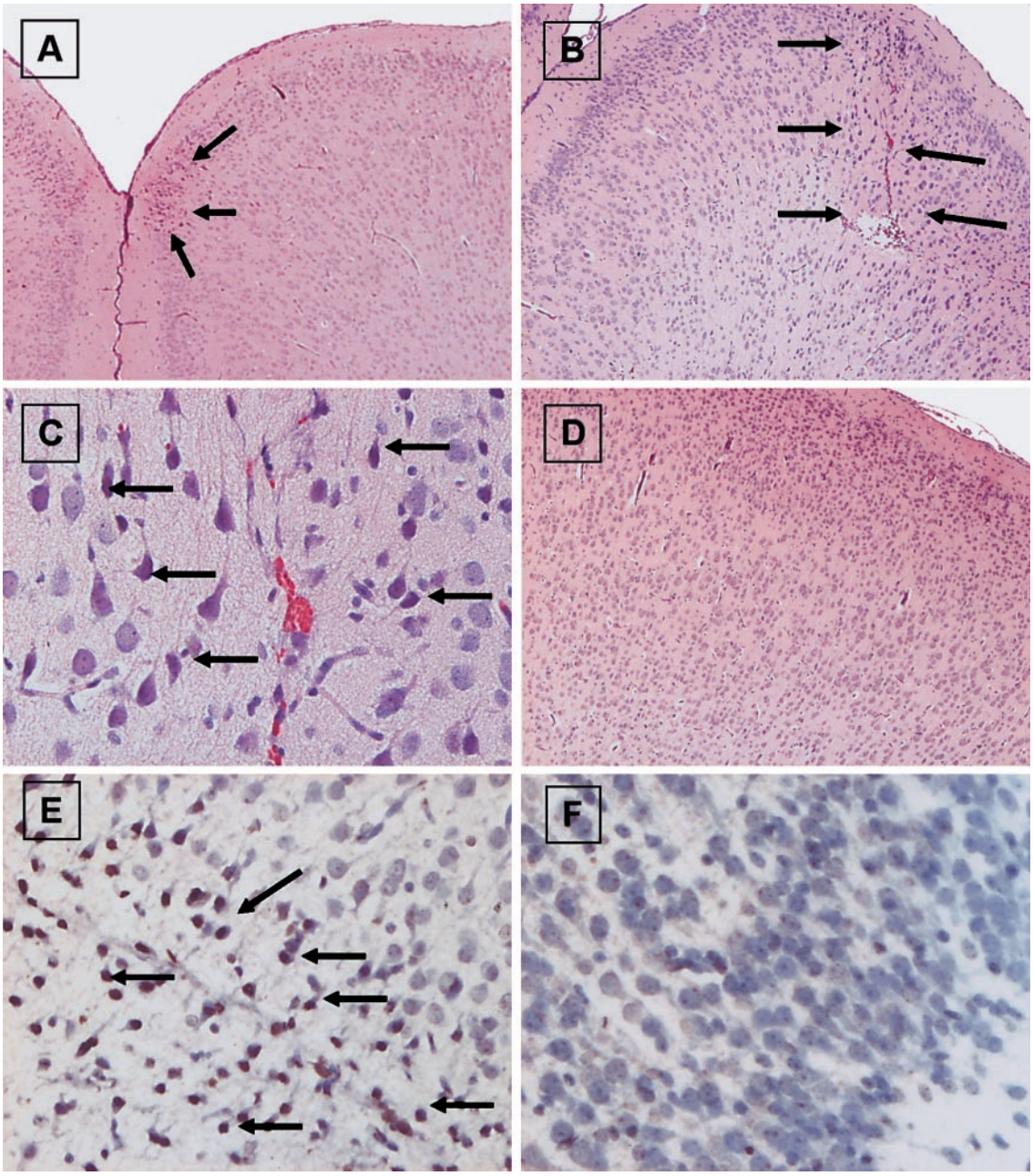


**Fig. 1.** Photomicrographs illustrating histopathological lesions resulting from prenatal exposure to LPS alone (**A**), or in combination with postnatal H/I (**B**, **C**). Coronal sections at P8 from the right frontal telencephalic hemisphere (**A**) showing foci of dark/shrunken neocortical neurons (arrows) often with hypereosinophilic neuronal cytoplasm. Severer lesions prevail in the neocortical gray matter (**B**, **C**). These were induced when prenatal LPS exposure was combined with neonatal H/I (group 4). **B** These lesions are characterized by the presence of an architectural disorganization that extends across most of the depth of the neocortex, together with the development of foci of microcavitation. The basic feature of the neocortical neuronal injury is also reproduced in these 'double-hit' brains: **C** The same shrunken/dark neurons with a hypereosinophilic perikaryonal cytoplasm. **D** No morphological changes were observed in the brain from our control group (group 1). **E** Coronal section of the right frontal cortex at P8 showing apoptotic neurons displaying TUNEL-positive nuclei (arrows) in a rat pup submitted to H/I (group 3). **F** TUNEL-negative nuclei in the frontal cortex at P3 in a control rat pup (group 1). Original magnifications: ×40 (**A**, **D**); ×100 (**B**); ×200 (**C**, **E**, **F**).

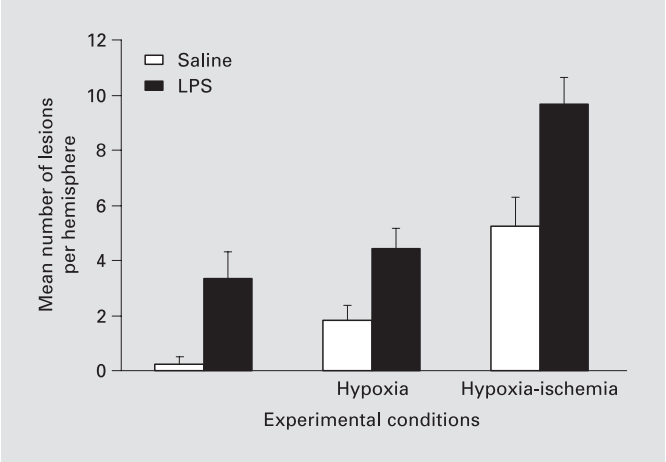


**Fig. 2.** Comparison between the mean numbers of lesions per cerebral hemisphere in the different experimental conditions. Exposure to hypoxia, or H/I alone, induced a significant increase in the number of brain lesions per hemisphere as compared to control conditions ($p < 0.01$); H/I also induced a significant increase in the number of brain lesions per hemisphere as compared to hypoxia ($p = 0.01$). Additional prenatal exposure to LPS significantly increased the number of brain lesions in all of our experimental conditions: LPS alone versus control ($p < 0.01$), LPS plus hypoxia versus hypoxia alone ($p < 0.01$), LPS plus H/I versus H/I alone ($p < 0.01$). Exposure to LPS plus H/I significantly increased the number of brain lesions as compared to LPS alone ($p < 0.01$) and to LPS plus hypoxia ($p < 0.01$). Data are from brains analyzed at P3 and P8.

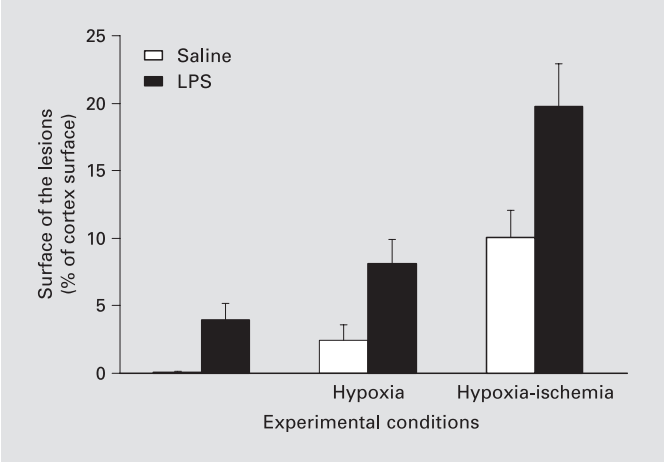


**Fig. 3.** Comparison of the surface of brain lesions in the different experimental conditions. Brains prenatally exposed to LPS present a significantly more extended surface of lesions as compared with control brains ($p < 0.01$). The total surface area of brain lesions induced by LPS exposure followed by hypoxia or H/I is significantly greater than the surface area of brain lesions induced by hypoxia ($p < 0.01$) or H/I ($p = 0.03$) alone; LPS plus H/I also induced a significant increase in the surface of brain lesions per hemisphere as compared to LPS plus hypoxia ($p < 0.01$). Hemispheres exposed to hypoxia, or H/I alone, present a significantly more extended surface of lesions as compared to control conditions ($p = 0.02$); H/I also induced a significant increase in the surface of brain lesions as compared to hypoxia ($p = 0.02$). Exposure to LPS plus hypoxia or H/I significantly increased the area of brain lesions as compared to LPS alone ($p < 0.03$). The proportion of lesions was calculated by measuring the surfaces of affected areas; the percentages were obtained by dividing figures for the lesioned surface area by the total surface area of the neocortex in the ipsilateral hemisphere. The surface of the lesions was measured on one coronal section from each group, all located according to classical stereotaxic coordinates between the interaural line 6.70 and 4.48 mm, and between the bregma 2.30 and 4.52 mm. Data are from brains analyzed at P3 and P8.

tected in the cerebral cortex of frontal, parietal and occipital lobes, and in amygdaloid and hypothalamic nuclei. The parasagittal regions of the frontal lobe were particularly affected namely the retrosplenial agranular cortex, cingular cortex, and the hindlimb and forelimb areas. These neuropathologic changes consisted principally of foci of dark, shrunken neuronal cell bodies and nuclei, mainly in the superficial layers of the cortex at P3 and P8. However, some of these lesions sometimes extended to the deepest cortical layers. In these affected areas, neuronal cells were generally hypereosinophilic. Apoptotic cell death was demonstrated in these affected cortical areas using the TUNEL method (fig. 1). Whereas some topographical variations in lesions were observed in different animals, they all, however, remained generally confined to the same cortical areas. There was a significant difference in the number and the extent of brain lesions between LPS-exposed rats (group 2) and control animals (group 1) (fig. 2, 3). There were no detectable histological changes in the cerebral white matter, cerebellum or brainstem.

*Neuropathologic Alterations Induced by Neonatal H/I (Group 3) as Compared to Control Group (Group 1)*

No clinical anomaly was detected in this experimental condition either. Brain examination at P3 and P8 showed no visible macroscopic anomaly. The brain weights of rat pups at P3 and P8 were identical in the H/I group (group 3) to those in the control experimental condition (group 1) (table 1). Microscopically, the right telencephalic hemispheres of newborn rats subjected to H/I (group 3) showed remarkable histopathological lesions in the gray matter. These were principally observed in the parasagittal regions of the frontal (retrosplenial agranular cortex, cingular cortex, hindlimb and forelimb areas), parietal and occipital cortices. These lesions at P3 were characterized by

shrinkage of neuronal cell bodies, condensation of cell nuclei, marked hypereosinophilic staining of affected neuronal perikarya, and disorganized neural cell architecture. Most often, the affected areas had a columnar pattern across the superficial layers of the neocortex or sometimes spanning the full thickness of the cortex. The TUNEL method demonstrated apoptotic cell death in most of the neurons of these affected cortical areas. At P8, the basic neuropathological picture was very similar to that observed at P3. However, some lesions at this later stage were characterized by the additional appearance of a few cortical microcavitational lesions. Both at P3 and P8, neuronal changes also affected deep brain nuclei in the right telencephalic hemispheres subjected to H/I, namely, thalamic and hypothalamic neurons. No visible histopathological changes were detectable in the hippocampus, white matter, cerebellum, or brainstem subjected to H/I. The number and the extent of lesions observed in the left telencephalic hemispheres subjected to hypoxia alone were significantly less than seen in right hemispheres subjected to combined H/I (fig. 2, 3). No histological alteration was detected in control animals (group 1).

*Neuropathologic Alterations Induced by the Combination of LPS and H/I (Group 4) as Compared to H/I alone (Group 3)*

In both groups (groups 3 and 4), and at the time of evaluation, no clinical anomaly was observed in rat pups subjected to this experimental procedure as compared with control animals. Thus, motor and feeding behavior, daily monitored for 30 min, from P1 to P8 did not reveal any visible anomaly. Macroscopic examination of the brain performed at P3 and P8 in pups submitted to LPS and H/I (group 4) showed no visible anomaly. At P8, the mean brain weight of pups was significantly lower in animals exposed to LPS plus H/I (group 4) as compared to those subjected to H/I alone (group 3) (table 1). Microscopic examination showed that prenatal exposure to LPS resulted in a significant increase in the number and the extent of cerebrocortical lesions induced by hypoxia (left hemisphere, group 4) or H/I (right hemisphere, group 4) (fig. 2, 3) as compared to the same experimental conditions but without LPS exposure (group 3). Affected areas of the brain in group 4 displayed mostly a columnar pattern mainly within the superficial cortical levels, or sometimes extending across most of the cortical depth (fig. 1). Lesioned areas were mainly located in the parasagittal regions of the frontal, parietal, and occipital cortices, and in amygdaloid nuclei. Foci of neuronal injury were also detected in some other deep gray structures, namely the hypothalamus and thalamus. The hindlimb, forelimb and the agranular retrosplenial areas of the frontal cortex presented extended lesions in most right hemispheres subjected to combined LPS and H/I. Whereas prenatal exposure to LPS in left hypoxic or right H/I hemispheres resulted in more extensive cerebrocortical lesions, the histopathological characteristics, and the profile of neuronal cellular changes were not, however, very different from those observed in hemispheres submitted to the same experimental conditions but without LPS exposure (group 3). Thus, at P3 and P8, lesions were characterized by foci of neuronal perikaryonal hypereosinophilia, shrinkage of cell bodies, and pyknotic or fragmented nuclei. The TUNEL method demonstrated apoptotic cell death in most of the neurons of these affected cortical areas. None of the experimental conditions in any group resulted in extensive infarcts. In group 4, no detectable lesions were observed in the cerebral white matter, hippocampus, cerebellum, or the brainstem.

## Discussion

We describe an animal model for perinatal brain lesions in which we showed that prenatal exposure to LPS resulted in cerebral gray matter changes, and amplified brain lesions induced by early neonatal H/I insults.

Our experimental design largely replicates the model originally developed by the Vanucci group [Rice et al., 1981] and subsequently used by other teams [Yager et al, 1991; Towfighi et al., 1994; Hagberg et al., 1998; Benjelloun et al., 1999]. Our modified protocol entailed H/I at an earlier neonatal stage, namely P1, as it had previously been performed in some studies using Sprague-Dawley rats [Sheldon et al., 1996; McQuillen et al., 2003]. The brain damages we observed were relatively similar to those described in these previous studies except for the induction of lesions within the hemisphere submitted to anoxia alone which was not described by others. This difference might be related to the use of distinct rat strains or to other slight differences between both experimental designs. Another characteristic of our experimental design as compared to others was to set out conditions simulating a supervened prenatal infection compounded with a later developing H/I disorder by introducing prenatal exposure to LPS from E17 to E20. Our objective was to replicate as far as possible the sequence of a prenatal infectious aggression and a subsequent neonatal H/I disorder. This sequence in the human is suspected to

be one of the main pathophysiological determinants leading to neonatal brain damage and ensuing lifelong disabilities, such as cerebral palsy, and cognitive and behavioral impairments. The cellular/architectural characteristics of brain lesions we observed in pups subjected to combined LPS-H/I insults (group 4) were reminiscent of the so-called pattern of selective neuronal necrosis characterizing some human neonatal encephalopathies occurring in many term and premature newborns [Volpe, 2001; Kinney and Armstrong, 2002; Yager, 2004]. Thus, the microscopic appearance of the neuronal cellular changes and the topographic localization of affected areas in our experimental model bear great resemblance to cortical lesions described in the human brain with these injuries. Based on these similarities, and on the reproducibility of the experimental protocols we designed, we think that our experimental setup could provide a suitable model that enables to more readily explore the various aspects of the pathophysiologic processes activated in these perinatal encephalopathies, particularly their infectious/inflammatory components and the various cellular/molecular interactions involved therein. However, in contrast with brain lesions observed in most human premature newborns, we did not histologically detect any visible damage affecting the white matter. Such absence of gross pathological lesions in the white matter does not necessarily exclude white matter injury, as it was previously described in other rat models studying the effect of H/I and LPS on brain development [Cai et al., 2000; Eklind et al., 2001; Bell and Hallenbeck, 2002; Yang et al., 2004]. Further experiments have to be conducted with our model to look for possible white matter lesions that might not be visible with hematoxylin-eosin staining. These should include the search for selective oligodendrocytic death, discrete astrogliosis, and microglial infiltration using immunohistochemical and other molecular techniques.

Our results showed that LPS regularly administrated intraperitoneally to pregnant rats, between E17 and E20, induced brain lesions which consisted of multiple foci of discrete neuronal necrosis mainly located in the neocortex (group 2). The topographic distribution and the cellular characteristics of the brain lesions induced by such an immune/inflammatory response to a systemic infection in rats bear much resemblance to those induced by H/I insults, but are slightly less severe when LPS is given alone. Various designs to explore the effect on the neonatal brain of LPS administration into pregnant animals have already been attempted. Cai et al. [2000] thus used a high dose of LPS (500 μg/kg) administrated intraperitoneally in two shots, at E18 and E19, in Sprague-Dawley rats. Bell and Hallenbeck [2002] tried *intrauterine* injection of one shot of LPS (100 μg/kg) at E15 and studied its effect on fetal (E20) and postnatal (P21) brain development in Lewis and Fisher 344 rats. Findings in these studies depict a consequent apoptotic cell death and hyperexpression of inflammatory cytokine (TNF-α) in white and/or gray matter of the cerebral hemispheres [Cai et al., 2000]. Besides, focal aggregates of reactive astrocytes were reported in the superficial layers of the cerebral cortex in locations corresponding to those where we observed foci of neuronal degeneration [Bell and Hallenbeck, 2002]. Studies on LPS exposure in other animal models [Debillon et al., 2000; Duncan et al., 2002; Garnier et al., 2003; Mallard et al., 2003; Peebles et al., 2003] provided further evidence in support of the potential deleterious effect on the developing brain exerted by such an exposure. Several procedural differences in the setting up of the experimental work among the various groups might have resulted in the subtle variation in results depicting the detailed histopathological findings. Outcome might also have been influenced by the animal species used in the various studies, i.e. some of the former studies were carried out in ovine, rabbits or rats. Among the procedural elements that could influence outcome severity or the extent of lesions are differences in the route of administration, dose, and timing of LPS exposure between the various studies.

Hence, it is likely that the level and/or the duration of maternal, and/or fetal innate immune system activation in response to LPS exposure might modulate the intensity of perinatal brain damages. In our experimental procedure, we tried to simulate a prenatal infectious condition as far as possible by maintaining a rather homogeneous exposure to endotoxin through repeated injections of LPS in regular intervals, and in a nonlethal dose. We designed to replicate in our model the potentially deleterious interaction between infectious processes and H/I conditions which are believed to play a crucial role in the pathogenesis of human perinatal brain damages. We showed that prenatal exposure to LPS significantly exacerbates the extent of cerebrocortical lesions induced by later supervening early postnatal H/I in the rat brain. Further, the sequential order and the timing of coupling the neurobiological effects of combined infectious H/I disorders in our design makes it a suitable model for further explorations aiming at investigating the cellular and molecular basis of many human cerebral lesions inflicted in the neonatal period. It is worth reminding that the LPS-induced sensitization of the newborn brain to a subsequent H/I insult has already been shown in the *postnatal*

period. Coumans et al. [2003], thus, showed that intracisternal LPS administration sensitized the 7-day-old Wistar rat brains to subsequent H/I brain injury. Besides, Eklind et al. [2001] found that LPS administered at a low dose to 7-day-old Wistar rats induced massive cerebral infarction following the exposure to relatively slight H/I. These findings are in line with our results and lend further support to the concept of a 'double hit' on the developing neonatal brain inflicted by compound infectious and hypoxic/ischemic insults. In addition, our findings show that this deleterious interaction could also occur when infection is initiated before birth. In this context it is worth noting that prenatal infections that could adversely affect the developing brain might have their origin in the maternal environment, and not necessarily within the fetal/placental compartment.

The precise nature of the pathophysiologic mechanisms implicated in the causation of the compounded effects of LPS in H/I conditions remains unknown. It has been suggested that the effect of LPS may be mediated through circulatory collapse and acidosis [Dalitz et al., 2003; Duncan et al., 2002; Garnier et al., 2003; Peebles et al., 2003]. These views, however, remain controversial. Thus, recent studies did not find support for the hypothesis that brain damage in newborn rats exposed to systemic LPS is due to reduced blood flow [Eklind et al., 2001; Coumans et al., 2003]. These results rather suggest that LPS damages the brain by some mechanism other than ischemia. Other studies outlined that LPS or ensuing systemic proinflammatory cytokines could act directly on the brain. This was supported by observations showing that mutations of the Toll-like receptor 4 on microglia inhibit LPS-induced oligodendroglial cell death [Lehnardt et al., 2003]. This would suggest a direct role for LPS on brain cells but should not rule out additional and independent hypoxic injury.

## Acknowledgements

We are very grateful to Liliane Brassard and Emilie Lareau Trudel for their help. This work is supported by grants from Sherbrooke University and 'La Fondation pour la Recherche sur les Maladies Infantiles', QC, Canada.

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