

# Combination of lipopolysaccharide and hypoxia-ischemia attenuates cholinergic anti-inflammatory effect in newborn rat brains

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## Summary

**Aim:** Pharmacologic parasympathetic nerve stimulation markedly reduces brain damage in a newborn rat model of hypoxia-ischemia (HI). Our aim is to determine whether a brain-protective effect can be achieved by parasympathetic nerve stimulation even with the synergy effect of lipopolysaccharide (LPS) and HI. **Study design:** 7-day-old Wistar rats were used. Rats received intraperitoneal administration of LPS which was followed by left carotid artery ligation. After a two-hour recovery period rats were placed in a hypoxic environment (8% oxygen) for 1 hour. Carbachol, used as an acetylcholine receptor agonist, or saline was injected subcutaneously immediately prior to one-hour hypoxia to determine its neuroprotective effect. Seven days later, the degree of brain damage in the ligated side of the hemisphere was compared to that in the contralateral hemisphere, which acts as reference. The relative difference in hemisphere area ((ligated side/non-ligated side) × 100%) was employed as an indicator of brain damage. Microglial aggregation (cells/mm<sup>2</sup>) on the cortex and cytokine production in the ligated side of the hemisphere were also evaluated. **Results:** Three pups died in the carbachol group. There was no significant difference in brain damage between the saline (n = 34) and carbachol (n = 30) groups (86.0 ± 12.1% vs. 90.8 ± 13.6%, respectively). There was no significant difference in microglial aggregation between the saline and carbachol groups (155.7 ± 201.1 cells/mm<sup>2</sup> vs. 89.8 ± 149.1 cells/mm<sup>2</sup>, respectively). IL-1β production in the saline and carbachol groups was 1.8 ± 1.2 pg/dL and 1.8 ± 1.1 pg/dL, respectively, with no significant difference between the groups. **Conclusion:** Combination of LPS and HI attenuates cholinergic anti-inflammatory effect in newborn rat brains.

**Key words:** Lipopolysaccharide; Inflammation; Hypoxia-ischemia; Parasympathetic nerve stimulation; Brain damage; Newborn rat.

## Introduction

Infection and inflammation during pregnancy threatens the healthy development of the fetus, where lung injury, brain injury, cerebral palsy (CP) and autism are the most prevalent detrimental outcomes [1]. In terms of inflammation-related brain injury, it has been known that chorioamnionitis increases the incidence of intraventricular hemorrhage (IVH) and CP [2]. Especially when inflammatory changes on the fetus side are severe, brain injury such as IVH can easily develop [3, 4]. Additionally, the synergistic effect of inflammation and hypoxia-ischemia (HI) causes increased brain damage [5, 6]. Thus, the establishment of an effective management strategy in the case of hypoxia-inflammation-related perinatal brain injury is urgent. Unfortunately, there are no effective measures at present to reduce hypoxia-inflammation-related perinatal brain damage.

We previously showed that pharmacologic parasympathetic nerve stimulation markedly minimized whole brain damage in a well-established newborn rat model of perinatal HI, where stimulating the parasympathetic nerve markedly minimized both microglial aggregation and cytokine production in the immature brain [7, 8, 9, 10, 11]. Our experiments were performed based on the finding of

parasympathetic nerve modulation of the inflammatory response in peripheral tissues [12, 13] and demonstrated the magnitude of the influence of neuroinflammation on perinatal brain damage after HI. Even in animal models of sepsis, parasympathetic nerve modulation of the inflammatory response suppressed cytokine production [14].

We previously conducted experiments of HI-related perinatal brain damage supposing a synergy effect of LPS and HI to induce inflammation, with the former (LPS) leading to increased brain damage [5]. It was also shown that therapeutic hypothermia failed to reduce perinatal morbidity [15]. However, it was yet to be determined whether pharmacologic parasympathetic nerve stimulation can minimize perinatal brain damage, which is induced by the synergy effect of inflammation and HI. We supposed that parasympathetic nerve stimulation is efficient in minimizing brain damage even in the presence of pathogen, i.e. LPS.

Therefore, we undertook investigations to determine the effect of pharmacologic parasympathetic nerve stimulation on tissue damage, microglial aggregation, and cytokine production resulting from the synergy effect of LPS and HI to induce inflammation in the immature brain.

## Materials and Methods

### Animal model

This study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Miyazaki (approval number: 2016-512-3) and conducted in keeping with the Guidelines of the Experimental Animal Center of the University of Miyazaki. The management of animals was conducted as follows. Pregnant Wistar rats were housed in the same animal center with free access to water and food under a 12-hr on/off lighting schedule. Rat pups were kept with their dams until the time of the experiment. Seven-day-old rats, whose cerebral maturity corresponded to a 23-36-week gestation human fetus [16], were subjected to Levine-Rice preparation [17, 18] for evaluation of brain damage. In this model, brain damage is usually limited to the ligated side of the cerebral hemisphere and the contralateral cerebral hemisphere represents the undamaged control. Use of 2, 3, 5-triphenyltetrazolium chloride (TTC) to stain hypoxic-ischemic lesions of Levine-Rice preparations revealed that lesions were limited to the ligated side of the hemisphere for up to 72 hours [19].

In the current experiment, we injected LPS intraperitoneally (1 mg/kg) to pups 4 hours prior to left carotid artery ligation. Pups were then anesthetized with isoflurane and the left common carotid artery was doubly-ligated with 5-0 surgical silk. Pups were then allowed to recover for 2 hours in the incubator without their dams. Carbachol, used as an acetylcholine receptor agonist, was administered subcutaneously (0.1 mg/kg,  $n = 33$ ) immediately prior to one-hour hypoxia to examine the brain-protective effect of carbachol with reduced microglial aggregation and production of cytokine. The dose of carbachol was determined from our previous experiment using pups and showed a potent effect in reducing brain damage following 2 hours hypoxia using the same experimental model employed for HI [7, 8, 9]. Pups in another group were given an equivalent volume of saline ( $n = 34$ ) immediately prior to one-hour hypoxia to compare the brain damage and were then exposed to hypoxia for one hour. Normally, brain damage in Levine-Rice preparations is not observed with hypoxia for one hour. A short hypoxic load comprising one hour was employed to examine the synergy effect with LPS. Pups in both groups were exposed to hypoxia in a chamber filled with humidified mixed gas comprising 8% oxygen and nitrogen at 33 °C. This temperature is the usual ambient temperature that pups are exposed to when huddling with dams [20]. Following hypoxia, pups were returned to their dams.

### Evaluation of brain damage

Pups were sacrificed on day 14 by a lethal dose of pentobarbital (100 mg/kg). The brains were removed and then fixed overnight in a 19 : 1 solution of ethanol and acetic acid, dehydrated, and then embedded in paraffin. The brain tissue of each pup was cut with a section 6  $\mu\text{m}$  in thickness that contained the dorsal hippocampus. Each coronal section was stained with hematoxylin-eosin. Assessment

of brain damage was performed as previously described [9, 10, 11]. Briefly, the area of the ligated side (left) was divided by the area of the non-ligated side (right) to obtain a ratio as the relative difference in hemisphere area ((ligated side/non-ligated side)  $\times 100\%$ ), which served as an index of brain damage. A ratio of 100% indicates no atrophy, while 0% indicates total atrophy.

### Immunohistochemical staining and measurement of microglial aggregation

Microglial aggregation of the cortex in the ligated side of the hemisphere was evaluated by tomato-lectin staining. Each thin section was washed with PBS and blocked with 0.5% bovine serum albumin for 30 minutes prior to immunohistochemical staining with biotinylated *Lycopersicon esculentum* tomato lectin (1 : 100) (Vector Laboratories) for 1 hour. Avidin binding was performed using a Vectastain ABC kit and developed using 3,3'-diaminobenzidine as a peroxidase substrate (Vector Laboratories). Sections were then rinsed in PBS, dehydrated in graded ethanol, cleared in xylene, mounted, and images were then acquired. Stained microglia show either branched (ramified) or amoeboid forms. The change in microglia from ramified to amoeboid forms is dependent on the activation pathway of microglia [21]. Amoebic microglia always show a stronger affinity for tomato-lectin compared to ramified microglia [22]. Amoeboid microglia are also more spherical in shape compared to ramified microglia [9, 10]. Firstly, we surrounded a measurement area in the cortex, measured the area ( $\text{mm}^2$ ), and then digitally omitted the region outside of the surrounded area (Figure 1A). Secondly, we identified amoeboid microglia by utilizing the difference in gradation and shape of stained microglia (Figure 1B), isolated the amoeboid microglia in the surrounded area, and then counted the number of amoeboid microglia using Image J software. This number was expressed as cells per  $\text{mm}^2$ .

### ELISA for IL-1 $\beta$

IL-1 $\beta$  production of the ligated hemisphere side was measured on day 14. Rats (carbachol 0.1 mg/kg,  $n = 16$ ; saline,  $n = 18$ ) were sacrificed, the brain was instantly separated into 2 hemispheres, and the ligated hemisphere was homogenized in N-PER neuronal protein extraction reagent (Thermo Scientific, IL, USA) containing protease inhibitors (Thermo Scientific Halt Protease Inhibitor Cocktail). Homogenates were centrifuged at 10,000 rpm for 10 minutes at 4 °C. Supernatants were stored at -30 °C until use. IL-1 $\beta$  protein levels were determined using Rat IL-1 $\beta$  Quantikine ELISA Kit (R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions. The optical density (OD) of reaction product was recorded using a microplate reader at 450 nm. The concentration of IL-1 $\beta$  protein was calculated from a standard curve. Each protein concentration was expressed as pg/mg total protein. Total protein concentration in all tissue was determined using a BCA protein Assay reagent Kit (Thermo Scientific, IL, USA).

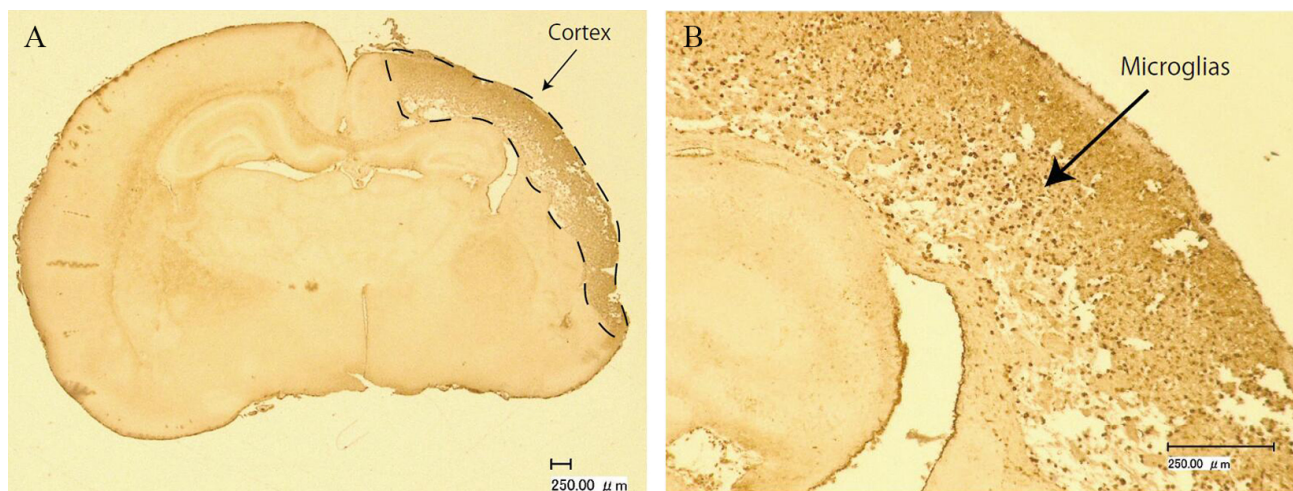


Figure 1. — Microglial aggregation on the cortex. A, The cortex region for assessment of microglial aggregation after hypoxic-ischemia (Tomato-lectin stain). B, Severe microglial aggregation was observed in the ligated side (left) cortex region (higher magnification, Tomato-lectin stain).

#### Statistical analyses

Results are expressed as the incidence or mean  $\pm$  SD. Comparisons between groups were made using the unpaired *t*-test. Probability values  $< 0.05$  were considered significant.

#### Results

##### Gross brain damage

Three pups died in the carbachol group. Thirty-four and 30 pups from the saline and carbachol groups, respectively, were used for the assessment of brain damage on day 14. As shown in Figure 2, there was no significant difference in the relative difference in hemisphere area between the saline ( $86.0 \pm 12.1\%$ ) and carbachol ( $90.8 \pm 13.6\%$ ,  $p = 0.14$ ) groups.

##### Microglial aggregation

Figure 3 shows microglial aggregation of cortex on day 14. Carbachol treatment had a lower suppressive effect on microglial aggregation ( $89.8 \pm 149.1$  cells/mm<sup>2</sup>) compared to saline treatment ( $155.7 \pm 201.1$  cells/mm<sup>2</sup>,  $p = 0.15$ ).

##### IL-1 $\beta$ production

IL-1 $\beta$  production of the ligated hemisphere side on day 14 is shown in Figure 4. IL-1 $\beta$  production in the saline and carbachol groups was  $1.8 \pm 1.2$  pg/dL and  $1.8 \pm 1.1$  pg/dL, respectively, with no significant difference between the groups ( $p = 0.95$ ).

#### Discussion

In our previous study that utilized a combination of LPS and intermittent HI in 7-day-old rats, brain damage was found in cases where heart rate variability was reduced during intermittent HI [23]. Heart rate variability is indicative of parasympathetic nerve activity [24]. We also showed that pharmacologic parasympathetic nerve stimulation significantly reduced whole brain damage in a rat model of perina-

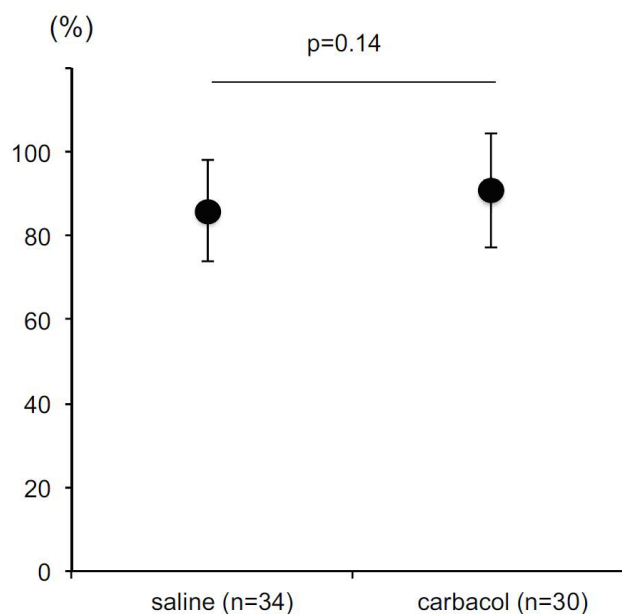


Figure 2. — Comparison of brain damage. Evaluation of the degree of brain damage was made by the relative difference in hemisphere area. Of note, there was no difference in brain damage between the saline and carbachol groups on day 14. The unpaired *t*-test was used to test for group differences. Data are expressed as mean  $\pm$  SD (%).

tal HI, where stimulation of parasympathetic nerve activity markedly reduced both microglial aggregation and cytokine production in the brain [8, 10, 11]. These observations account for the importance of neuroinflammation as a cause of HI-related tissue damage in the immature brain and for the magnitude of involvement of parasympathetic nerve activity which controls neuroinflammation that is caused by HI. In an adult animal model, parasympathetic nerve mod-

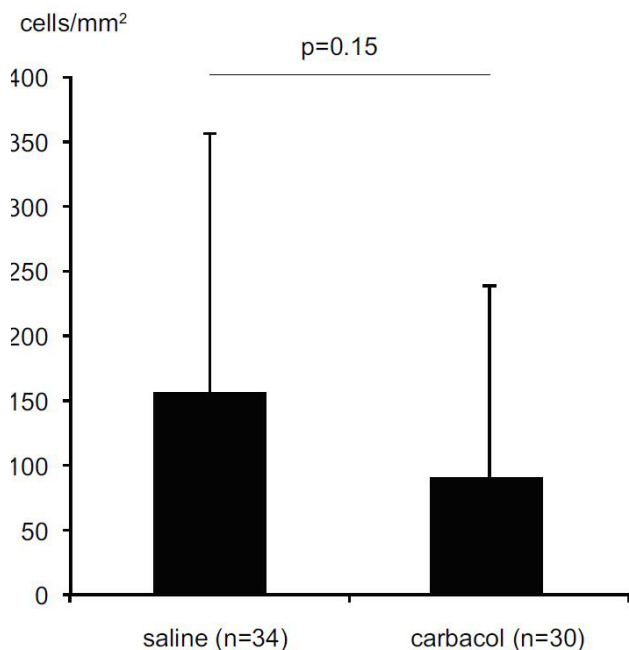


Figure 3. — Comparison of microglial aggregation on the cortex. We measured the number of microglia in the ligated side of cortex. Of note, there was no difference in microglial aggregation between the saline and carbachol groups on day 14. The unpaired *t*-test was used to test for group differences. Data are expressed as mean ± SD (microglia cells/mm<sup>2</sup>).

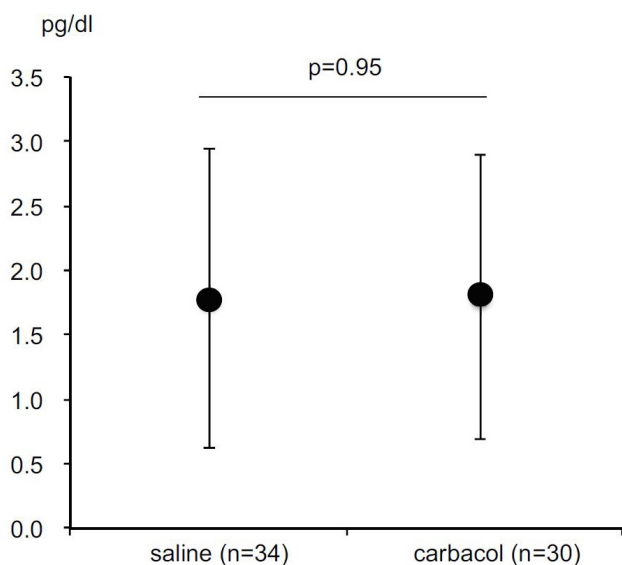


Figure 4. — Comparison of concentration of IL-1β. There was no difference in concentration of IL-1β between the saline and carbachol groups on day 14. A comparison of groups was made using the unpaired *t*-test. Data are expressed as mean ± SD.

ulation of the inflammatory response is efficient even when sepsis is present [14]. However, it has yet to be shown that pharmacologic parasympathetic nerve stimulation can re-

duce perinatal brain damage, which is induced by the synergy effect of inflammation and HI. Therefore, we hoped to suppress brain damage caused by the combination of LPS and HI in a newborn rat model. However, parasympathetic nerve stimulation failed to reduce brain damage in the current experiment.

The mechanism of brain tissue damage induced by the combination of inflammation and HI is partially due to increased production of cytokines such as IL-1β, IL-6, IL-8, and TNFα [25, 26]. LPS activates Toll-like receptor 4 (TLR4)-dependent vulnerability via triggering of the MyD88-dependent pathway, which leads to NF-κB-dependent production of IL-1β and TNFα [27]. Parasympathetic nerve modulation of the inflammatory response in peripheral tissues has been recognized [12, 13]. Stimulation of the α7 nicotinic acetylcholine receptor (α7nAChR) suppresses cytokine production via inhibition of NF-κB nuclear translocation and results in decreased cytokine production and tissue protection [28]. We also showed that pre-treatment with galantamine, an acetylcholinesterase inhibitor, significantly reduced the production of IL-1β after HI in the immature brain [11]. Therefore, it should be possible to suppress brain damage even in the presence of a synergy effect according to the theory. Recently, the mechanism of resistance against parasympathetic nerve modulation of the inflammatory response was revealed in the presence of LPS in an *in vitro* study, where decreased α7nAChR in the mouse brain was followed by exacerbating chronic inflammation [29, 30, 31]. These observations could account for the fact that the effect of parasympathetic nerve modulation was not recognized in our current study. Unlike other tissues, in the presence of pathogen such as LPS, parasympathetic nerve modulation of the inflammatory response may not work well in the immature brain.

Our study has some limitations. Since galantamine has more of a brain-protective effect without aggregation of microglia compared to carbachol, it is necessary to conduct experiments using galantamine in an effort to examine the anti-inflammatory effect of the parasympathetic nerve. A promising effect in reducing brain damage could be expected following treatment with galantamine. We have not examined changes in α7nAChRs. Furthermore, we were unable to present an effective method to reduce brain tissue damage caused by the synergy effect of LPS and HI to induce inflammation in the immature brain. Given that the synergistic effect resulting from inflammation and HI causes increased brain damage in actual clinical practice [6], further research is required.

In conclusion, we demonstrated that the synergy effect of LPS and HI, which exacerbates inflammation, reduces the brain-protective effect of parasympathetic nerve stimulation in the newborn rat. Under conditions that include the synergy effect of inflammation and HI, there is presently no effective strategy to control neuroinflammation of immature brain.

## Data Availability

The experimental data used to support the findings of this study are available from the corresponding author upon request.

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## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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