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Experimental paper

Hypothermia is not neuroprotective after infection-sensitized neonatal hypoxic–ischemic brain injury☆

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ABSTRACT

Background: Therapeutic hypothermia (HT) is the standard treatment after perinatal hypoxic–ischemic (HI) injury. Infection increases vulnerability to HI injury, but the effect of HT on lipopolysaccharide (LPS) sensitized HI brain injury is unknown.
Design/methods: P7 rat pups were injected either with vehicle or LPS, and after a 4 h delay they were exposed to left carotid ligation followed by global hypoxia inducing a unilateral stroke-like HI injury. Pups were randomized to the following treatments: (1) vehicle treated HI-pups receiving normothermia treatment (NT) (Veh-NT; $n = 30$); (2) LPS treated HI-pups receiving NT treatment (LPS-NT; $n = 35$); (3) vehicle treated HI-pups receiving HT treatment (Veh-HT; $n = 29$); or (4) LPS treated HI-pups receiving HT treatment (LPS-HT; $n = 46$). Relative area loss of the left/right hemisphere and the areas of hippocampi were measured at P14.
Results: Mean brain area loss in the Veh-NT group was $11.2 \pm 14\%$. The brain area loss in LPS-NT pups was $29.8 \pm 17\%$, which was significantly higher than in the Veh-NT group ($p = 0.002$). The Veh-HT group had a significantly smaller brain area loss ($5.4 \pm 6\%$), when compared to Veh-NT group ($p = 0.043$). The LPS-HT group showed a brain area loss of $32.5 \pm 16\%$, which was significantly higher than in the Veh-HT group ($p < 0.001$). LPS-HT group also had significantly smaller size of the left hippocampus, which was not found in other groups. LPS-sensitization significantly decreased the sizes of the right, unligated-hemispheres, independent of post-HI treatment.
Conclusions: Therapeutic hypothermia is not neuroprotective in this LPS-sensitized unilateral stroke-like HI brain injury model in newborn rats. Lack of neuroprotection was particularly seen in the hippocampus. Pre-insult exposure to LPS also induced brain area loss in the unligated hemisphere, which is normally not affected in this model.

1. Introduction

Perinatal hypoxic–ischemic (HI) injury is one of the major causes of long-term neurological disability or death in term newborns. Infection is known to be a major confounding factor for neonatal morbidity and mortality, especially in developing countries.[1]

Perinatal infection increases the vulnerability of the newborn brain to HI.[2–5] In an established neonatal rat model of unilateral HI brain injury,[6] a longer period of exposure to hypoxia results in increased brain injury.[7] In the same model, increased injury can also follow a shorter period of hypoxia, if animals have been subjected to a mild infectious stimulus such as pre-exposure to bacterial lipopolysaccharide (LPS) before HI.[3]

Therapeutic hypothermia (HT) is the standard treatment for term infants after perinatal HI injury,[8] as it has been shown to significantly reduce mortality and neurodevelopmental disability in survivors.[9] However, around 50% of cooled asphyxiated newborns still suffer poor outcomes,[9] some of which may have been exposed to perinatal infection.[10] It has been shown that HT influences many cascades that follow a HI injury, including inflammatory and anti-inflammatory pathways,[11–13] but this influence might be different following pre-exposure to an infectious stimulus.

Abbreviations: CNS, central nervous system; HI, hypoxia–ischemia; HT, therapeutic hypothermia; i.p., intraperitoneal; LPS, lipopolysaccharide; NT, normothermia; SD, standard deviation.

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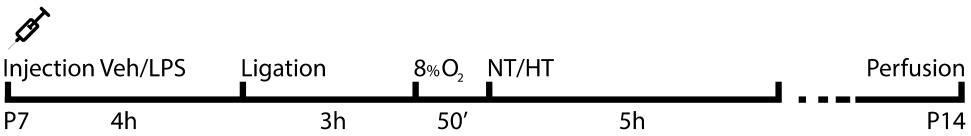

Fig. 1.

This study was designed to investigate the neuroprotective effect of HT in a double-hit neonatal model of LPS-sensitized HI brain injury, which has not been previously investigated.

2. Material and methods

2.1. Procedures

All experiments were approved by the University of Oslo's animal ethics research committee. Experiments were performed on 7 day old (P7) Wistar rats (Charles River, Sulzfeld, Germany) of both sexes, randomized across litter, sex and weight. All pups were kept in an animal facility with a 12:12 h dark/light cycle at 19–21 °C environmental temperature with food and water ad libitum, and were weighed and checked for health daily.

At the start of every experiment, we injected the animals according to randomization with either a single i.p. injection of vehicle solution (0.9% NaCl) or LPS solution (*Escherichia coli* O55:B5, Sigma; 0.1 mg kg^{-1}). After a 4 h delay, some of the animals were exposed to HI accordingly. We have used the established "Vannucci HI Rat Model".[6] Briefly, under general isoflurane and N_2O anesthesia, one common carotid artery (the left in our experiments) is ligated and cut. Within 3 h the pups are subjected to hypoxia. We and others typically use 90 min of 8% O_2 at 36 °C in neuroprotection studies.[14,15] Using a validated neuropathology score, this insult results in 50–60% injury on the ligated side only,[16] or 40% area loss (left hemisphere/right hemisphere) when imaged sections are digitally analyzed.[7] In this study we chose a milder insult of 50 min instead of 90 min of 8% O_2 at 36 °C, as it was known a priori that adding LPS would increase injury.[3] Pups were sacrificed on postnatal day P14 (Fig. 1).

2.2. Effect of an intraperiotoneal (i.p.) injection of vehicle or LPS without HI

To show that a single i.p. injection of vehicle solution or LPS solution did not have an effect on mortality or brain pathology per se, 30 P7 rat pups received either a single dose of vehicle ($n = 10$), LPS ($n = 10$), or none of the above (juvenile control, $n = 10$). The control pups were not ligated and were not exposed to hypoxia, but after injections, all pups were returned to dams for 7 h followed by 5 h at 37 °C in a temperature controlled chamber flushed with air to simulate the timing and the experimental conditions used in the HI experiments that followed. Pups were then returned to their dams and sacrificed at P14.

2.3. Lipopolysaccharide sensitized model of hypoxic–ischemic brain injury

A total of 140 P7 rat pups of both sexes from 14 litters were used. Pups from all litters were randomized to the following four groups: (1) Vehicle treated pups receiving normothermia treatment (NT) (Veh-NT; $n = 30$); (2) LPS treated pups receiving NT treatment (LPS-NT; $n = 35$); (3) vehicle treated pups receiving HT treatment (Veh-HT; $n = 29$); or (4) LPS treated pups receiving HT treatment (LPS-HT; $n = 46$). As we knew from previous experiments that animals treated with LPS had a higher mortality during hypoxia, we randomized more animals into the LPS treated groups, so that the final numbers of animals in all groups would be similar.

According to the randomization, pups received either a single dose of vehicle ($n = 59$) or LPS (0.1 mg kg^{-1} i.p.; $n = 81$), given in a volume of 10 μl g^{-1} of body weight. After a 4 h delay the pups were exposed to a mild HI insult.

Immediately after the HI insult, pups received either of the 2 allocated treatments: 5 h of NT (T_{rectal} 37.0 °C) or HT (T_{rectal} 32.0 °C). During treatment, core temperature was continuously recorded in each chamber in two "sentinel" pups, which were from one of the vehicle injected groups (either Veh-NT or Veh-HT), carrying a rectal temperature probe (IT-21, Physitemp Instruments, Clifton, NJ, USA). Temperature of the chamber was maintained within ±0.2 °C of the target value using a continuous rectal probe temperature recording, which servo-controlled the mat (CritiCool, MTRE, Yavne, Israel). Rectal temperature correlates within 0.1 °C with brain temperature in P7 rats.[17] For the HT group, a T_{rectal} of 32.0 ± 0.2 °C was achieved within 15 min after transfer of the pups to the new chamber. After the treatment period, pups were returned to their dams and sacrificed at P14.

2.4. Histopathology and area measurement

After seven days of survival, at P14, transcardiac perfusion with 10% neutral-buffered formalin was performed under isoflurane/N_2O-anesthesia. Brains were harvested and kept in 10% neutral-buffered formalin for 7 days until further processing. Coronal 3 mm blocks were cut through the brain using a standard matrix (ASI Instruments Inc., Warren, MI, USA) and embedded in paraffin. Five μm sections were stained with hematoxylin and eosin (H&E). Two sections from each of the two neighboring blocks best representing cortex, hippocampus, basal ganglia and thalamus, were scanned with a virtual microscopy scanner (Axio Scan.Z1, Carl Zeiss, Jena, Germany) using brightfield mode with plan apochromatic 20× lens. Virtual slides were exported as 600 dpi images. To measure the area of brain tissue loss, the image of each section was analyzed with ImageJ software (ImageJ, version 1.46r, National Institutes of Health, Bethesda, MD, USA) and assessed by an individual who was blinded to group allocation as previously described.[7] The average percentage of area loss was calculated from the two neighboring sections by using the following formula: (1 − (area left/area right)) × 100 (Fig. 2).

We separately evaluated the areas of the contralateral (unligated) hemispheres (which had intact blood supply; Fig. 3) and the hippocampal areas of both hemispheres (Fig. 4). For the analysis of unligated hemispheres only, the sizes of hemispheres were corrected for individual body weight of the rat pups at P14, as we have used absolute hemispheric sizes for this analysis, and the brain size could be influenced by the feeding ability of the pups. The difference in the size of hippocampi (not corrected for body weight) was calculated as: (1 − (area of left left hippocampus/area of right hippocampus)) × 100. A subset of the H&E stained sections were examined for hemispheric and hippocampal areas by two blinded assessors to check for inter-rater reliability.

2.5. Data analysis

Statistical analyses were performed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA). One way ANOVA was used to compare the different treatment groups, followed by a non-parametric

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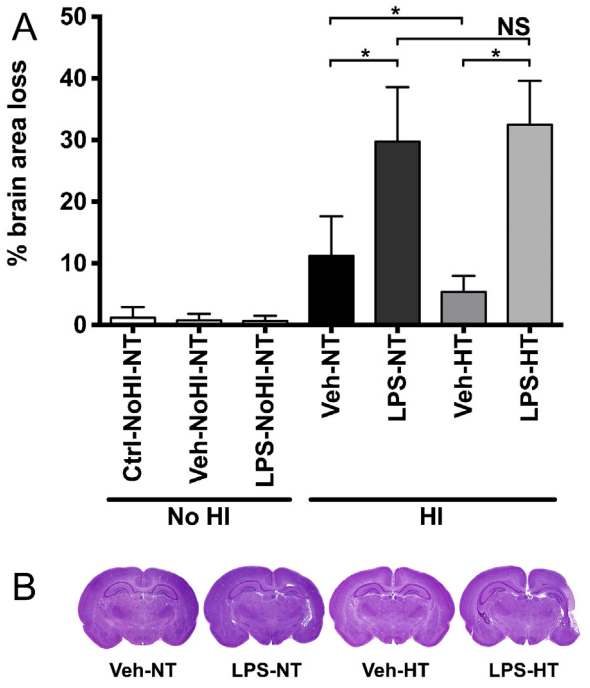

Fig. 2. (A) Brain area loss regarding treatment. Animals that were injected with LPS had a significantly higher brain area loss compared to those that were injected with vehicle, regardless of the post-HI treatment (NT or HT). HT significantly reduced brain area loss in animals injected with vehicle, while failed to do so in animals injected with LPS. (B) Representative images of brain tissue belonging to animals of various treatment groups. Left hemispheres lie on the right side of images. Ctrl-NoHI-NT: juvenile control pups, not ligated, no HI, treated with NT, $n = 10$; Veh-NoHI-NT: vehicle injected pups, not ligated, no HI, treated with NT, $n = 10$; LPS-NoHI-NT: LPS injected pups, not ligated, no HI, treated with NT, $n = 10$; Veh-NT: vehicle treated pups receiving NT treatment, $n = 22$; LPS-NT: LPS treated pups receiving NT treatment, $n = 18$; Veh-HT: vehicle treated pups receiving HT treatment, $n = 24$; LPS-HT: LPS treated pups receiving HT treatment, $n = 24$; $^*p < 0.05$; NS: not significant. Boxes: mean, bars: 95% CI.

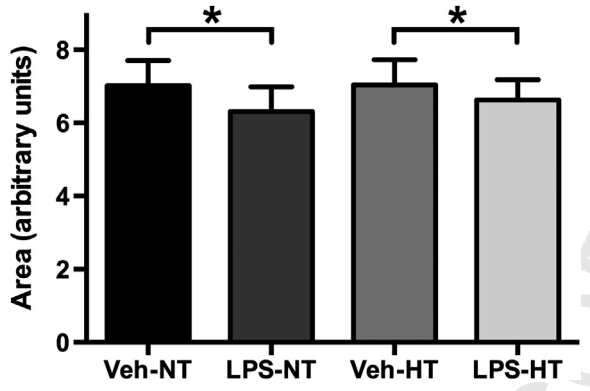

Fig. 3. Area of unligated right hemispheres. LPS significantly decreased the size of unligated hemispheres in both NT and HT treated animals. This finding suggests that hypoxia alone can be detrimental in LPS sensitized rat pups, whereas normally a combination of HI is required to cause brain injury. Veh-NT: vehicle treated pups receiving NT treatment; LPS-NT: LPS treated pups receiving NT treatment; Veh-HT: vehicle treated pups receiving HT treatment; LPS-HT: LPS treated pups receiving HT treatment; boxes: mean; bars: 95% CI; $^*p < 0.05$.

post hoc test (Tamhane) if the ANOVA showed significant differences between treatment groups. The Wilcoxon–Mann–Whitney test was used for two-group comparisons. Effects of sex and P7 weight of pups on brain area loss and the effect of HT and LPS on the size of unligated hemispheres were estimated by linear regression. The p value of <0.05 was considered statistically significant.

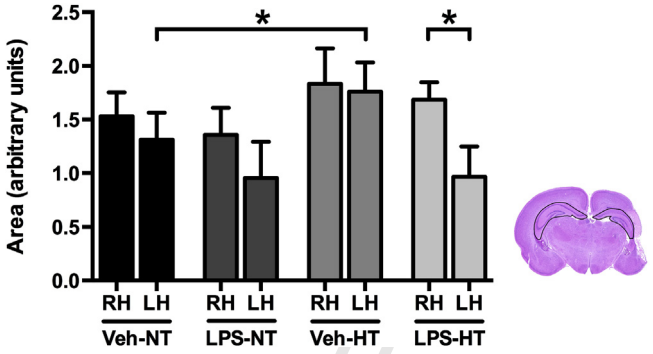

Fig. 4. Area of hippocampi of the right (unligated) and left (ligated) hemisphere. Left: the hippocampi of the left hemisphere (exposed to HI) were smaller than those of the right (exposed to hypoxia only) in all groups. The difference was bigger in animals injected with LPS, compared to those injected with vehicle in both NT and HT treated animals. The left hippocampi were significantly smaller than the right in the LPS-HT group ($p < 0.001$), whereas this difference was not significant in any other group. The difference between the left and right hippocampus was smallest and the absolute sizes of hippocampi biggest in the Veh-HT group, showing the neuroprotective effect of HT. Right: a scan of brain tissue from one of the animals of the LPS-NT group outlining the hippocampal area that was measured using ImageJ. Left hemisphere lies on the right side of the image. Veh-NT: vehicle treated pups receiving NT treatment; LPS-NT: LPS treated pups receiving NT treatment; Veh-HT: vehicle treated pups receiving HT treatment; LPS-HT: LPS treated pups receiving HT treatment; boxes: mean; bars: 95% CI upper limit; $^*p < 0.01$.

Descriptive data are presented as mean ± standard deviation (SD). Graphical data are presented as mean + 95% confidence interval (CI).

3. Results

Of 140 rat pups, 6 pups died during ligation, 3 between ligation and the hypoxic insult, 21 during the 50 min of hypoxia, 1 during the 5 h treatment period and 9 between P8 and P14. Of the 9 that died after the treatment period, 7 received LPS (2 in the NT LPS group and 5 in the HT LPS group). The twelve sentinel pups carrying temperature probes were excluded from further analysis, because the stress of carrying the probe could influence the outcome.[18] The final number of animals included in analysis of brain area loss was 88: (1) Veh-NT, $n = 22$; (2) LPS-NT, $n = 18$; (3) Veh-HT, $n = 24$; and (4) LPS-HT, $n = 24$.

There was no significant difference between the 4 groups regarding sex and weight at P7 ($p = 0.76$ and $p = 0.11$). Linear regression showed no significant effect of sex and weight at P7 on brain area loss ($p = 0.17$ and $p = 0.41$). At P14, both NT and HT treated pups that received LPS gained significantly less body weight compared to the ones that received vehicle ($p = 0.007$ and $p < 0.001$; Table 1).

3.1. A single i.p. injection of either vehicle or LPS did not induce brain injury without HI

As shown in Fig. 2, a single injection of vehicle or LPS without HI did not result in brain injury or mortality, as all pups survived until P14. The juvenile control group had a L/R hemispheric area ratio of 1.2 ± 2% (mean ± SD), the vehicle injected pups 0.8 ± 1% and the LPS injected pups 0.6 ± 1%. Differences in L/R hemispheric ratio were not significant between these groups.

3.2. LPS sensitized model of HI brain Injury

LPS increased both mortality and morbidity of the pups exposed to HI brain injury. Mortality of rat pups was significantly higher in LPS injected pups, regardless of the therapy after HI. In the Veh-NT group 92.9% of the pups survived to P14, compared to 57.6% of the pups in the LPS-NT group ($p = 0.002$). In Veh-HT group 96.6% of the

Table 1
Sex, weight at P7, weight at P14 and brain area loss across different groups. Values given as mean ± SD (range).

	Veh-NT	LPS-NT	Veh-HT	LPS-HT	Total	*p*
Sex f/m	15/15	18/17	15/14	19/27	67/73	.76
Weight P7 (g)	10.2 ± 1.5 (6.5–13.0)	10.3 ± 1.3 (7.2–13.0)	10.6 ± 1.1 (8.5–13.1)	10.9 ± 1.1 (8.2–13.7)	10.5 ± 1.3 (6.5–13.7)	.11
Weight P14 (g)	24.1 ± 3.4 (18.7–31.3)	21.1 ± 3.5 (14.8–28.1)	24.4 ± 2.8 (19.1–29.1)	19.5 ± 2.8 (14.5–25.1)	22.4 ± 3.7 (14.5–31.3)	<.001
Brain area loss (%)	11.2 ± 14 (0–51.95)	29.8 ± 17 (0–56.87)	5.4 ± 6 (0–21.23)	32.5 ± 16 (0–60.95)		<.001

pups survived, compared to 59.1% of the pups in the LPS-HT group ($p < 0.001$).

There was a significant increase in brain area loss in the pups that received LPS, compared to those who received vehicle, in both NT and HT treated pups (Fig. 2). The mean left hemispheric brain area loss in the Veh-NT group was 11.2 ± 14%. The brain area loss in LPS-NT pups was 29.8 ± 17%, which was significantly higher than in the Veh-NT group ($p = 0.002$). The Veh-HT group had a significantly lower brain area loss (5.4 ± 6%), when compared to Veh-NT group ($p = 0.043$). This shows, that HT reduced brain area loss in vehicle treated animals in this mild hypoxic insult model. The LPS-HT group had a brain area loss of 32.5 ± 16%, which was significantly higher than in the Veh-HT group ($p < 0.001$). Thus, after NT treatment, pups injected with LPS had a 2.7-fold higher brain area loss, compared to those injected with vehicle. Following HT treatment, the pups injected with LPS had a 6.1-fold increase in brain area loss, compared to those injected with vehicle. Therapeutic hypothermia did not exhibit a neuroprotective effect in LPS sensitized pups.

3.3. *Effect of LPS on the unligated hemispheres and the size of the hippocampi*

Comparing the sizes of the unligated hemispheres (right side) across different groups, we found that pups that received LPS (regardless of whether they were treated with NT or HT) had reduced size of the unligated hemispheres, compared to pups treated with vehicle (Fig. 3). Assuming normal distribution of the area data after adjustment to individual body weight, we used linear regression with the two treatments as potential explanatory variables and found that HT treatment did not influence the size of the unligated hemispheres, but LPS significantly decreased their size. Since both the mean area and the SDs were similar in the Veh-NT group and the Veh-HT group and also similar in the two LPS groups (Fig. 3), we combined the groups pairwise and tested the potential effect of LPS treatment using the non-parametric Wilcoxon–Mann–Whitney test. The linear regression and the two-sample test gave similar results. LPS caused a significant decrease in brain area of the unligated hemispheres within the significance level of 5% ($p = 0.043$; one-tailed test). As we knew a priori from previous data[3] that LPS enhances vulnerability in the developing brain, using a one-tailed test is appropriate.

When comparing the size of left hippocampi to the size of the right across different groups, the differences in hippocampal areas were as follows: in Veh-NT group the difference was 15.8 ± 21%, in LPS-NT group 38.6 ± 31%, in Veh-HT group 6.3 ± 7%, and in LPS-HT group 45.7 ± 29% (Fig. 4). As expected, the left hippocampi were smaller than the right in all groups. However, this difference was significant only in the LPS-HT group ($p < 0.001$), exposing the fact that HT was not neuroprotective in this group. On the other hand, HT did exhibit a neuroprotective effect in the vehicle treated pups, as the left hippocampi in the Veh-HT group were significantly bigger than the left hippocampi of the Veh-NT group ($p = 0.016$). Furthermore, in the Veh-HT group the differences between the left and right hippocampi were smallest and the absolute sizes of left and right hippocampi were biggest, compared to other groups.

A subset of hippocampal measurements ($n = 28$) was also checked for inter-rater reliability giving a Pearson's correlation coefficient of 0.92, $p < 0.001$.

4. Discussion

We have shown that sensitizing a neonatal unilateral HI brain injury model with LPS injection prior to the HI injury increases the injury about 3-fold at NT. The new finding of the current study is that HT is not neuroprotective after LPS-sensitized HI brain injury as compared to HT without LPS.

Therapeutic hypothermia is one of the most intensively studied neuroprotective strategies to date. It is recommended by the ILCOR guidelines for two conditions, adult out of hospital cardiac arrest[19] and newborn hypoxic–ischemic encephalopathy.[8] Hypothermia has also been studied after stroke[20,21] and brain and spinal cord trauma,[22] where large randomized controlled trials have either not been undertaken or have failed to show efficacy. HT affects many pathways that are activated after central nervous system (CNS) injury including excitotoxicity, free radical production and inflammation, thereby reducing associated apoptotic and necrotic cell death as well as disturbances in cerebral blood flow regulation, metabolism and blood–brain barrier integrity.[23–25] As shown in different animal models, HT has a large suppressive effect on inflammation,[11,12] which might serve as one of its major protective mechanisms.

As reported by Covey et al., inflammatory processes occur almost immediately after tissue damage to the CNS.[26] The injured brain stimulates innate immune responses leading to activation of microglia and circulating leukocytes, and these immune cells can then release various molecules, including reactive oxygen species, proteases and pro-inflammatory cytokines, leading to a vicious cycle of cell death and immune activation.[27,28] Suppressing neuroinflammation is generally neuroprotective in animal models of developmental brain injuries.[23,26] However, some of the inflammatory processes are necessary for tissue repair and recovery from CNS injury.

LPS is a bacterial endotoxin that has been used in different animal models and experimental settings to induce inflammation in the preterm and term brain. This was first studied by Eklind et al., who have shown that a single injection of LPS (0.3 mg kg^{-1} i.p.) does not induce brain injury or glial activation.[3] They also found that the combination of a single injection of LPS combined with a mild hypoxic–ischemic injury dramatically increases brain injury.[3] In this two hit model of LPS sensitized hypoxic–ischemic brain injury we have used a lower dose of LPS (0.1 mg kg^{-1}) and still our findings were in accordance with those described by Eklind et al. LPS treated pups have been generally sicker, which was demonstrated by higher mortality, smaller weight gain and a more pronounced brain injury, compared to vehicle treated pups.

We have shown that HT is not neuroprotective in this scenario, which is a finding that has potentially important implications for neuroprotective treatment with HT in animal models and human newborns. The neuroprotective effect of HT was completely lost in animals that were sensitized with LPS, as suggested by the fact that HT failed to reduce the injury to the left hemisphere in the LPS-HT group of animals. Furthermore, Monje et al. and Hoehn et al.

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previously described that LPS-induced inflammation decreased the proliferation of precursors in the hippocampal subgranular zone, depressing hippocampal neurogenesis.[29,30] It has been shown that HI is more damaging to hippocampi than hypoxia alone,[31] and our study shows that this effect was more pronounced in animals that were injected with LPS and treated with HT. LPS-HT animals had significantly smaller left hippocampi (exposed to HI) compared to right hippocampi (exposed to hypoxia only), which was not the case in LPS-NT animals, or any of the groups of animals injected with vehicle. This effect is probably underestimated in our study, as the hemispheres with intact blood supply also showed a trend to be more injured if the animals were injected with LPS. LPS also significantly decreased the size of unligated hemispheres, which suggests that hypoxia alone can be detrimental in LPS sensitized rat pups.

As mentioned before, HT has been shown to suppress inflammation. Our findings suggest that this might not be beneficial in the infection-sensitized model of neonatal HI brain injury. The exact mechanisms by which HT modulates the inflammatory processes and why these changes are so detrimental in LPS sensitized neonatal model of unilateral HI brain injury are not known yet. One possible explanation might be that HT negatively influences the already activated microglia or that HT reduces some of the beneficial anti-inflammatory cytokines.

A limitation to this study is the focus on macroscopic brain injury only. Future investigation into the underlying molecular and cellular mechanisms will be essential in order to fully explain the observed findings. However, due to the potentially important clinical implications, we feel it is beneficial to present the initial data ahead of more detailed mechanistic work. In addition, LPS-stimulated inflammation does not necessarily mimic all of the inflammatory processes induced by a bacterial infection in the neonatal period. In a clinical setting, subsequent antibiotic administration could also influence the process. As a pro-inflammatory stimulus, it is possible that LPS may increase any hemodynamic compromise caused by HI or subsequent HT. While this is actively monitored and treated in human neonates undergoing HT, it is not technically possible to monitor this in a small newborn rodent model. Further research ought to be directed toward understanding the inflammatory processes that influence HT neuroprotection.

An additional new finding of this study using a mild-hypoxia model is that HT nevertheless was neuroprotective in animals that were not sensitized with LPS. This has potentially important clinical implications, as many newborns are exposed to mild degree of hypoxia, but there are currently not clear guidelines on how to treat this population of newborns. Further studies on neonatal exposure to mild hypoxia and treatment with hypothermia are warranted.

In a clinical scenario, chorioamnionitis is one of the major risk factors leading to preterm birth and long-term neurological disabilities. As reported by Thayyil et al.[32] and Lawn et al.[1] infection and perinatal asphyxia remain one of the major risk factors for under-5 year mortality in developing countries. As suggested in the recent meta analysis, newborns that have been exposed to both chorioamnionitis and perinatal asphyxia do not seem to profit from HT.[9] Moreover, a recent multicenter, randomized clinical trial of moderate HT in adult patients with severe bacterial meningitis by Mourvillier et al., had to be stopped early because HT was found to increase mortality compared to NT controls.[33] Our current study also calls into question the viability of HT after inflammatory sensitized HI injury in rat pups.

Conflict of interest statement

I declare that neither me or any of the co-authors are in conflict of interest.

Disclosures

None.

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