**Therapeutic hypothermia for the treatment of neonatal hypoxia-ischemia: sex-dependent modulation of reactive astrogliosis**

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**Abstract**

Therapeutic hypothermia (TH) is the standard treatment for neonatal hypoxia-ischemia (HI) with a time window limited up to 6 h post injury. However, influence of sexual dimorphism in the therapeutic window for TH has not yet been elucidated in animal models of HI. Therefore, the aim of this study was to investigate the most effective time window to start TH in male and female rats submitted to neonatal HI. Wistar rats (P7) were divided into the following groups: NAÏVE and SHAM (control groups), HI (submitted to HI) and TH (submitted to HI and TH; 32ºC for 5 h). TH was started at 2 h (TH-2 h group), 4 h (TH-4 h group), or 6 h (TH-6 h group) after HI. At P14, animals were subjected to behavioural tests, volume of lesion and reactive astrogliosis assessments. Male and female rats from the TH-2 h group showed reduction in the latency of behavioral tests, and decrease in volume of lesion and intensity of GFAP immunofluorescence. TH-2 h females also showed reduction of degenerative cells and morphological changes in astrocytes. Interestingly, females from the TH-6 h group showed an increase in volume of lesion and in number of degenerative hippocampal cells, associated with worse behavioral performance. Together, these results indicate that TH neuroprotection is time- and sex-dependent. Moreover, TH started later (6 h) can worsen volume of brain lesion in females. These data indicate the need to develop specific therapeutic protocols for each sex and reinforce the importance of early onset of the hypothermic treatment.

**Keywords** Neonatal hypoxia-ischemia · Therapeutic hypothermia · GFAP · Hippocampus · Neurodevelopment · Astrocytes

**Introduction**

Neonatal hypoxia-ischemia (HI) is one of the main causes of mortality and morbidity in neonates (Nelson and Lynch 2004; Shankaran et al. 2005). Moreover, HI survivors can develop cerebral palsy, neurosensory deficits and cognitive impairments throughout life (Shankaran et al. 2005). Currently, therapeutic hypothermia (TH) is well established as the standard treatment used for neonates with moderate to severe injury caused by neonatal HI (Abate et al. 2021). TH must be started within a period of 6 h after the hypoxic event (therapeutic window) in order to achieve desired neuroprotective effects (Gunn et al. 1997; Cho et al. 2020). In humans, the recommended protocol for TH is reduction of the temperature of the newborn to 33–34ºC for 72 h (Davies et al. 2019). However, in experimental settings, the efficacy of hypothermia protocols seems to depend on the species studied (Gunn et al. 1997; Wood et al. 2016). A hypothermia...
of 30°C lasting 120 h showed the best neuroprotection in sheep when started 5.5 h after hypoxic-ischemic insult (Gunn et al. 1997). However, in rats, TH protocols using longer periods and lower temperatures do not show additional neuroprotection and can even increase brain injury (Sabir et al. 2012, 2014; Wood et al. 2016).

TH is used in clinics irrespective of patients’ sex (Shankaran et al. 2005; Cho et al. 2020). However, there is growing evidence of the existence of sexual dimorphism in neural responses following HI, especially in animal models (Netto et al. 2017); thus, it is conceivable that neuroprotective effects of TH could be influenced by sexual dimorphism. In fact, some studies in animal models of HI have shown that neuroprotective effects of TH may depend on animals’ sex (Thoresen et al. 2009; Burnsed et al. 2015; Smith et al. 2015, 2016; Nie et al. 2016). A better performance in behavioural tests was observed in females treated with TH as compared to males, whereas other studies found no sex-related differences on the volume of injury or the behavioural responses when sex was considered as a variable (Wagner et al. 2002; Sabir et al. 2012, 2014; Fang et al. 2013).

Together with microglia activation and peripheral leukocyte infiltration into the nervous tissue, reactive astrogliosis plays a key role in the extension of the injury following HI. Astrocyte hyperactivity is associated with secondary neuronal damage due to release of proinflammatory cytokines after HI (Ahn et al. 2018; Escartin et al. 2021). TH is able to reduce reactive astrogliosis (Sabir et al. 2016; Ahn et al. 2018); however, it is not clear whether this reduction is influenced by sexual dimorphism.

Clinical studies demonstrate that the therapeutic window for hypothermia in neonatology has been set to a maximum of 6 h after the hypoxic-ischemic event (Gunn et al. 1997; Davies et al. 2019; Catherine et al. 2021). However, there is no such definition in animal models of HI, with experimental hypothermia being applied during hypoxia or up to 12 h post-insult (Sabir et al. 2012; Burnsed et al. 2015; Smith et al. 2015; Reinboth et al. 2016). In order to better understand the mechanisms through which TH produces its beneficial effects, an experimental time-window shall be established.

In the present study, we aimed to characterize the effective time window of TH in male and female rats submitted to neonatal hypoxia-ischemia evaluating developmental (body weight, negative geotaxis and righting reflex), morphological (brain lesion volume and degenerating cells in the hippocampus) and structural (GFAP immunoreactivity and astrocyte morphology) parameters following HI. The working hypothesis is that the effects of therapeutic hypothermia are influenced by sexual dimorphism.

Materials and methods

Animals

In this study, 208 seven-day-old (P7) Wistar rats (male and female) and 26 dams obtained from the Animal Facility of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, RS were used. The pups were kept in 270 × 260 × 310 mm plastic boxes with their respective mothers (08 pups per box). All animals were kept in a 12 h/12 h light/dark cycle with controlled temperature (±22°C) and mothers received food and water ad libitum. This study was approved by the Institutional Animal Care and Use Committee of the Federal University of Rio Grande do Sul (#31,442).

Neonatal hypoxia-ischemia

Neonatal HI procedure was based on the Rice-Vannucci model (Rice et al., 1981) and our previous studies (Sanches et al. 2013a; Fabres et al. 2018, 2020a; Tassinari et al. 2020). At P7, under isoflurane anaesthesia (5% for induction and 3% for maintenance), neonates underwent surgical occlusion of the right common carotid artery. A longitudinal incision was made in the neck to allow access to the right common carotid artery which was isolated and permanently occluded using surgical thread (silk 4.0). After surgery, pups were left with their mothers for 1-2 h for recovery and then placed in a hypoxia chamber (n = 6 animals per chamber) and exposed to an atmosphere of a certified mixture of 8% O₂ and 92% N₂ for 90 min at 37 °C. After hypoxic exposure, pups were removed from the chamber and kept in a warm box, for approximately 15 min, and then returned to their mothers.

Therapeutic hypothermia

Animals were placed in an acrylic chamber inside a temperature-controlled water bath for the hypothermia procedure (water temperature: 18–20°C). The animals reached a body temperature of 32°C (standard temperature indicated for therapeutic hypothermia in newborn animals) (Wood et al. 2016) within 20 min. Animals that failed to reach this temperature within the initial 20 min were excluded from the experiment. Animals were maintained in the hypothermic chamber for 5 uninterrupted hours. After hypothermia, animals were rewarmed for 30 min until they reached a body temperature of 36–37°C and then returned to their mothers (Wood et al. 2016).

For this study three different therapeutic time windows to start HT after HI were used: hypothermia started two hours (TH-2 h group), four hours (TH-4 h) or six hours (TH-6 h)
after hypoxic-ischemic event. From end of hypoxia exposure until beginning of hypothermia, animals from each group were kept in their boxes with their mothers. For each time window (2, 4 or 6 h to start hypothermia) there were three respective control groups: NAÏVE group (animals not manipulated), SHAM group, and HI group (animals submitted to HI but kept in normothermia). These last two groups were used to monitor the differences caused by the time animals were kept away from their mothers. However, as no differences were observed among these control groups, they were combined into a single SHAM group. All experimental groups were additionally separated into males and females. Sixteen (16) animals were randomly allocated per group. For this study, a mortality rate of 7.69% of total animals was found (16/208). The timeline of the experiment is depicted in Fig. 1.

**Body temperature and body weight**

Body temperature of each animal during the experiments was measured using an infrared thermometer (Incoterm, TCI 1000). The accuracy of an infrared thermometer was compared to that of a rectal thermometer in a pilot study which showed differences lower than 0.5°C between them (data not shown). Thus, we decided to continue experiments using only the infrared thermometer to avoid the stress caused by the rectal one (Smith et al. 2015).

Animal’s body temperature was measured every 5 min during the first 20 min of hypothermia. All animals in the TH groups reached the temperature of $32 \pm 0.5$°C within these first 20 min. After that, body temperature of animals from groups SHAM, HI and TH was measured every 30 min. Temperature of animals in the NAÏVE group (kept in their own boxes) was not measured in order to reduce manipulation effects, as well as to reduce dam’s stress. Every animal was weighed at two timepoints: before surgery for carotid occlusion (P7) and before euthanasia (P14).

**Histological analysis**

In order to assess the volume of lesion of the cerebral hemisphere, hippocampus and cerebral cortex, animals (P14) were deeply anesthetized with isoflurane and transcardially perfused with saline solution (0.9% NaCl) followed by 4% paraformaldehyde (PFA). Brains were removed and submerged in a solution of 4% PFA overnight, then dehydrated in an alcoholic series (80%, 90%, 96% and 100% ethanol) and cleared in xylene. Afterwards, brains were embedded in paraffin, sectioned in the coronal plane (7 μm) using a microtome (Microm HM 340E ThermoScientific) and stained with hematoxylin and eosin (HE).

The following antero-posterior coordinates were used for brain analysis: +2.52 mm to -6.84 mm relative to bregma for analysis of the cerebral hemisphere; +1.70 mm to -4.16 mm for the cerebral cortex and −2.04 mm to -6.12 mm for the hippocampus. All coordinates were obtained according to the Paxinos and Watson rat brain atlas (Paxinos and Watson 2007) and to a previous study from our group (Fabres et al. 2020a). In order to reduce possible errors due to occurrence of brain oedema, the equation described by Sun et al. (2015) was used to calculate the volume of lesion (Sun et al. 2015; Fabres et al. 2020a).

The same images used to assess brain lesion were used to calculate a score indicative of lesion severity (n = 10), according to Thoresen and colleagues (Thoresen et al. 1996) by an evaluator blinded to the groups. Injury severity was divided into 9 levels varying from 0 (without injury) to 4 (maximum injury), with 0.5 intervals. The classification was performed based on the percentage of injury according to the scale: 0 (without lesion), 1 (injury equal to or less than 10%), 2 (indicative of 20–30% of lesion), 3 (injury between
40 and 60%) and 4 (more than 75% of injury). To calculate pathological score of the hippocampus, animals with percentage of lesion below or equal to 20% were classified with a score of 1; a score of 2 indicates 50% injury, a pathological score of 3 indicates 75% lesion; and a score of 4 indicates a value of 100% injury.

**Cell counting**

To estimate the number of cells in degeneration (cells showing shrinkage and deformity of the cell body or karyorhectic and pyknotic nuclei) we used slides stained with haematoxylin and eosin as described in a previous paper (Fabres et al. 2020a). CA1 subfields and hilus of the dentate gyrus of the hippocampus ipsilateral to the lesion were evaluated. Five different sections containing CA1 and hilus were evaluated per animal. The images were obtained using a microscope (Zeiss) at a magnification of × 400. Mean of number of degenerative cells divided by mean of total number of cells × 100 was used to estimate percentage of degenerative cells in each subfield.

**Immunofluorescence and morphological analysis**

Sections were deparaffinized using xylene (10 min) and rehydrated using a decreasing alcoholic series. After these procedures, staining was performed as previously reported (Fabres et al. 2020a). For identification of astrocytes, a primary anti-astrogial fibrillary acidic protein antibody (anti-GFAP, #G9269, rabbit IgG, 1:200, Sigma-Aldrich) was used and the secondary antibody of choice was Alexa 488 anti-rabbit (1:500, Molecular Probes, Invitrogen). The slides were sealed with mounting medium containing DAPI (Merck, #G9269, rabbit IgG, 1:200, Sigma-Aldrich) was used and the secondary antibody of choice was Alexa 488 anti-rabbit (1:500, Molecular Probes, Invitrogen). The slides were sealed with mounting medium containing DAPI (Merck, F6057) and coverslipped.

For analysis of fluorescence intensity (used as an indicative of reactive astrogliosis), 5 sections per animal were used (n = 5 animals per group); NAÏVE and SHAM groups were used as controls. All images were captured with high magnification (400 x) using a Nikon Eclipse E-600 microscope (Japan). A 3800 µm² area of optical interest was defined to delimit all areas of interest and value of integrated density per unit of area was obtained using ImageJ software (NIH, Bethesda, USA).

The assessment of astrocyte morphology (Fig. 2) in the CA1 area of the hippocampus (number of primary processes and length of processes relative to soma), was performed in 3 astrocytes per Sect. (5 sections per animal, totalling 15 astrocytes per animal). Sholl’s concentric circles method was used for this analysis: concentric circles with 2 µm intervals were drawn around each cell analyzed using Image-Pro Plus software 6.0 (Mestriner et al. 2011).

**Behavioural Assessment**

The test of negative geotaxis was performed according to Ahn and colleagues (Ahn et al. 2018) with minor modifications. At P14, each animal was placed individually on a platform with an inclination of 35°, the head turned towards the bottom of the platform. The latency of the animal to rotate 180° and position itself with its head facing top of the platform was registered (Ahn et al. 2018). Each animal was tested three times and the mean latency was calculated.

To test the righting reflex at P14, animals were removed from their cages and put on their back on a flat surface. The latency necessary to straighten up with all paws on the surface was recorded (Hermans et al. 1992).

**Data analysis**

All statistical analyses were performed using GraphPad Prism 6.0. Normality test was performed, and parametric data were analyzed by two-way analysis of variance (ANOVA) (factors: sex and group) followed by Bonferroni post-hoc test. Data were plotted as box-and-whiskers with boxes depicting median and 25th and 75th percentiles, and whiskers representing minimum and maximum values for each data set.

**Results**

**Body weight**

At P7, there were no differences in body weight among groups before animals underwent neonatal HI and two-way ANOVA did not detect effect of group (F5,176 = 0.07140, p = 0.9964) nor sex (F1,176 = 0.03382, p = 0.8543). However, at the age of 14 days, there was a significant effect of both factors, group (F5,176 = 20,56, p < 0.0001) and sex (F1,176 = 14,65, p = 0.0002) on body weight, but no interaction was verified (F5,176 = 0.4278, p = 0.8289). Animals submitted to HI showed significantly lower body weight as compared to control groups (p < 0.05), except for the TH-2 h group (p > 0.05), in which both (males and females) had body weights similar to animals from the control groups (Fig. 3).

**Brain injury volume**

Figure 4 A shows volume of lesion in the right cerebral hemisphere. Two-way ANOVA showed a significant effect of the group (F5,180 = 149.7, p < 0.0001), with no effect of sex (F1,180 = 1.626, p = 0.2050) on the volume of lesion. HI groups showed a volume of injury of the brain hemisphere as compared to NAÏVE and SHAM groups (p < 0.05). When
all groups submitted to HI showed an increased in cortical volumes of injury relative to the NAÏVE and SHAM groups \((p < 0.05)\). The TH-2 h group showed a significantly smaller volume of lesion as compared to the HI group \((p < 0.05)\), for both sexes. It was also observed that males from the TH-2 h group had a larger volume of lesion in the hemisphere compared to the TH-2 h female group \((p < 0.05)\).

Pathology score

Pathology scores of the cerebral hemisphere are shown in the Fig. 4D. There was a significant effect of group \((F_{5,180} = 455.7, p < 0.0001)\), and sex \((F_{1,180} = 5.025, p = 0.0270)\) and an interaction between both factors \((F_{5,108} = 2.506, p = 0.0346)\). The HI group showed an increased pathology score as compared to NAÏVE and SHAM groups \((p < 0.05)\).
sex (F_{1,180} = 7.032, p = 0.0092), as well as significant interaction between factors (F_{5,108} = 8.369, p = 0.0220). The HI group had a greater pathology score compared to NAÏVE and SHAM groups for both sexes (p < 0.05). In males, both TH-2 h and TH-4 h groups showed a reduction in pathology score relative to the HI group (p < 0.05). Among females, only the TH-2 h group showed a reduced score as compared to the HI group (p < 0.05). In addition, females from the TH-4 h group showed a higher pathology score in the cerebral cortex compared to the TH-4 h male group (p < 0.05).

**Percentage of degenerative cells**

The percentage of degenerative cells was analyzed in the CA1 and hilus subfields of the hippocampus ipsilateral to carotid occlusion (Fig. 5 A). In the CA1 region, two-way ANOVA detected a significant effect of group (F_{5,180} = 350.4, p < 0.0001) and sex (F_{1,180} = 14.22, p = 0.0003), as well as an interaction between factors (F_{5,108} = 8.369, p < 0.0001). The HI group showed a higher percentage of degenerative cells in relation to the NAÏVE and SHAM groups (p < 0.05). In males, the TH-2 h group showed a decrease in percentage of degenerative cells in CA1 relative to the HI group (p < 0.05). Among the females, only the TH-2 h group showed a reduced score compared to the HI group (p < 0.05). Moreover, the percentage of degenerative cells in the CA1 area of groups TH-2 h and TH-4 h was higher in females than in males (p < 0.05).

When percentage of degenerative cells was evaluated in the hilus of the dentate gyrus (Fig. 5 B), two-way ANOVA showed a significant effect of group (F_{5,180} = 350.4, p < 0.0001) and sex (F_{1,180} = 14.22, p = 0.0003), with an interaction between factors (F_{5,108} = 6.056, p < 0.0001). For both sexes, HI groups followed the same pattern observed in the CA1 area, showing an increase in percentage of degenerative cells as compared to the NAÏVE and SHAM groups (p < 0.05). In males, the TH-2 h group showed a decrease in percentage of degenerative cells in the hilus relative to the HI group (p < 0.05). Among the females, only the TH-2 h group showed a reduction in percentage of degenerative cells compared to the HI group (p < 0.05). However, a sex-related difference was also observed; in the HI and TH-2 h groups a smaller percentage of degenerative cells was observed in females (p < 0.05). Besides that, the TH-6 h female group showed an increase in percentage of degenerative cells in comparison to the HI female group (p < 0.05).
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of the length of processes of astrocytes (Fig. 6 C), two-way ANOVA detected a significant effect of group (F_{4,39} = 31.42, p < 0.0001), with no effect of sex (F_{1,39} = 1.865, p = 0.1799) when the number of primary process of astrocytes was evaluated (Fig. 6B). In the analysis of the length of processes of astrocytes (Fig. 6 C), two-way ANOVA detected a significant effect of group (F_{4,37} = 55.28 p < 0.3598) and sex (F_{1,37} = 0.8596 p = 0.3598).

Animals from both sexes showed an increase in length of the processes in the HI group as compared to the control group; however, only females from TH-2 h group had reduced length of the processes compared to the HI group (p < 0.05). Male and female HI groups showed the same pattern observed in analyses of the length of processes: there was an increase in number of astrocyte primary processes compared to the control group (p < 0.05) and TH treatment reduced the number of processes only in the TH-2 h female group as compared to the HI group (p < 0.05).

**GFAP immunoreactivity and astrocyte morphology**

GFAP immunoreactivity and astrocyte morphology were studied in the CA1 subfield of the hippocampus ipsilateral to the carotid occlusion (Fig. 6). Two-way ANOVA showed a significant effect of group (F_{4,40} = 19.87 p < 0.0001) and sex (F_{1,40} = 6.625 p = 0.00139) on GFAP fluorescence intensity (Fig. 6 A). The HI group showed an increase in GFAP fluorescence intensity as compared to the control group, for both sexes. Males from the TH-2 h and TH-4 h groups as well as females from TH-2 h showed reduction in GFAP fluorescence relative to the HI group (p < 0.05).

In addition, two-way ANOVA showed a significant effect of the group (F_{4,39} = 31.42, p < 0.0001), with no effect of sex (F_{1,39} = 1.865, p = 0.1799) when the number of primary process of astrocytes was evaluated (Fig. 6B). In the analysis by Bonferroni’s post hoc test and were depicted as box-and-whiskers plots. Boxes represent median and interquartile range (25th – 75th percentiles) and whiskers represent the minimum and maximum values for each data set (n = 10). *significant differences compared to the NAÏVE and SHAM groups (p < 0.05); *significant difference compared to the HI group (p < 0.05); #significant differences between males and females (p < 0.05). Bar = 0.5 cm

**Fig. 4 Upper panel:** images of brain coronal sections stained with hematoxilin and eosin representative of the lesion volume in the distinct experimental groups. **Lower panel:** percentage of volume of lesion of the cerebral hemisphere (A), hippocampus (B) and cerebral cortex (C) and pathology score of the cerebral hemisphere (D), hippocampus (E) and cerebral cortex (F), ipsilateral to carotid occlusion in males and females. Data were analyzed by two-way ANOVA followed by Bonferroni’s post hoc test and were depicted as box-and-whiskers plots. Boxes represent median and interquartile range (25th – 75th percentiles) and whiskers represent the minimum and maximum values for each data set (n = 10). *significant differences compared to the NAÏVE and SHAM groups (p < 0.05); #significant difference compared to the HI group (p < 0.05); #significant differences between males and females (p < 0.05). Bar = 0.5 cm
The analysis of the righting reflex showed a significant effect of group ($F_{5,180} = 25.55$ $p<0.0001$), with no effect of sex ($F_{1,180} = 0.2714$ $p=0.6030$); an interaction between the factors was also found ($F_{5,180} = 2.637, p=0.0250$). The results were similar to those found for the negative geotaxis test, i.e., animals from the HI group (from both sexes) showed an increase in latency to complete the righting reflex test. However, only males from the TH-2 h group reduced the latency as compared to the HI group. Moreover, females from the TH-6 h group showed an increase in latency to complete the test relative to the HI group (Fig. 7B).

**Correlation analysis**

Pathological score is used as a tool to estimate level of brain lesion following neonatal HI (Thoresen et al. 1996; Sabir et al. 2014). In order to assess the validity of our pathological score, we checked if these results were correlated with the results from the volume of lesion. There was a positive correlation between the pathological score and volume of lesion for all structures evaluated (cerebral hemisphere, hippocampus, and cerebral cortex) for both sexes ($p<0.05$; Table 1).

A positive correlation between the latency to complete behavioral tests and the volume of lesion or pathology score was also observed for all structures evaluated (cerebral hemisphere, hippocampus and cerebral cortex) for both sexes ($p<0.05$; Table 1).

The data of volume of lesion, pathological score and percentage of degenerative cells in the hippocampus showed a positive correlation with the level of reactive astrogliosis (mainly related to length of processes and number of primary processes) for both sexes ($p<0.05$; Table 1).

**Discussion**

In this study, we have shown that the effective time window to start TH is sex-dependent. TH initiated earlier, i.e., 2 h after injury, reduced extent of lesion in the cerebral hemisphere, hippocampus, and cerebral cortex for both sexes. However, the onset of hypothermia 6 h after hypoxia worsened the results of morphological (percentage of degenerative cells in the hippocampus) and behavioral parameters, mainly in females. The best results of TH are achieved when treatment starts immediately or shortly after the hypoxic-ischemic event (Sabir et al. 2012; Cho et al. 2020).

Body weight is a developmental parameter which can provide important information about the protective effects of hypothermia. It is well known that HI can lead to motor deficits which, in turn, lead to feeding difficulties and affect animal’s body weight gain (Sanches et al. 2013a; Fabres et
animals belonging to the TH-2 h group showed a decrease in lesion volume in every brain structure evaluated when compared to the HI group. However, when TH was started later (TH-4 h and TH-6 h groups), this neuroprotective effect of TH was blunted, suggesting TH started later may not be able to protect the brain properly. In agreement with Davson (2015) the cells can recover from an insult up to 6 h after the event and this period is known as the latent period. Within this period, deleterious mechanisms can be initiated leading to spread of brain injury and progressive cell dysfunction (Davidson et al. 2015; Cho et al. 2020).

There are experimental and clinical evidences showing that the closer the onset of hypothermia is to the moment of HI, the greater the reduction of brain injury (Sabir et al. 2012, 2014; Thoresen et al. 2013) observed that hypothermia started immediately or 3 h after HI caused a reduction in lesion volume in every brain structure evaluated when compared to the HI group. However, when TH was started later (TH-4 h and TH-6 h groups), this neuroprotective effect of TH was blunted, suggesting TH started later may not be able to protect the brain properly. In agreement with Davson (2015) the cells can recover from an insult up to 6 h after the event and this period is known as the latent period. Within this period, deleterious mechanisms can be initiated leading to spread of brain injury and progressive cell dysfunction (Davidson et al. 2015; Cho et al. 2020).

The lack of weight loss observed in the TH-2 h group may be associated with smaller volume of brain damage and, consequently, reduction in motor impairment. Here, by two-way ANOVA followed by Bonferroni’s post hoc and were depicted as box-and-whiskers plots. Boxes represents the median and interquartile range (25th – 75th percentiles) and whiskers represent the minimum and maximum values for each data set (n = 5). *significant differences compared to the control groups (p < 0.05); †significant differences compared to the HI group (p < 0.05). Bar = 100 μm

Fig. 6 Upper panel: representative images of GFAP immunofluorescence for each experimental group in the CA1 area ipsilateral to carotid occlusion. GFAP fluorescence intensity analysis (A), the length of the process leaving the soma (B) and the number of primary processes of astrocytes (C) were evaluated in the CA1 area of the hippocampus ipsilateral to carotid occlusion in males and females. Data were analyzed...
However, it is known that there is a difference in type of cell death depending on sex; in females, cell death is mainly dependent on caspase activation, whereas in males, cell death is mostly caspase-independent (Joly et al. 2004; Aska-lan et al. 2015; Netto et al. 2017).

These differences in cell death pathways may be one of the reasons why we observed an increase in lesion volume and in the number of degenerative cells in females, since the caspase pathway might have been still active at the time point the animals were evaluated (Fabres et al. 2020a, 2020b). Therefore, an evaluation at the cellular level could help us find differences between sexes. The hippocampus was chosen for this analysis, since it is the most affected brain structure in the neonatal HI animal model (Dhikav and Anand 2012).

When percentage of degenerative cells in pyramidal layer of CA1 and hilus was evaluated, a sex-related effect was revealed. Animals from the HI group showed an 8-fold increase in percentage of degenerative cells for both sexes, which is in accordance to our previous study using only males (Fabres et al. 2020a). TH reduced percentage of cells in degeneration when started up to 4 h after injury, although the most remarkable neuroprotection was observed in the TH-2 h group. In addition, in both hilus and CA1, the percentage of degenerative cells in females from the TH-2 h group was around 15%, while in the TH-2 h males this percentage was around 30% in hilus and 35% in CA1. These results corroborate with data showing that TH is able to reduce neuronal death in the CA1 area of the hippocampus three and seven days after injury (Xiong et al. 2009; Wood et al. 2016). The hilus of the dentate gyrus was chosen as being a region considered less vulnerable to HI than the CA1 area. Nevertheless, it was also shown that hypothermia started earlier was able to reduce cell degeneration in the hilus; on the other hand, when started 6 h after HI (TH-6 h group), the number of degenerating cells in females was even greater than that observed in the HI group.

Reduction in volume of brain injury and in percentage of degenerative cells are important factors indicative of the efficacy of TH; however, it is well known that brain injury caused by HI can extend for weeks to months (Davidson et al. 2015). Neuroinflammation is one of the main causes that extends the injury for longer periods. It is also known that male animals submitted to neonatal HI show increased microglial activation and upregulation of the inflammatory response (Netto et al. 2017). Astrocytes play a role in this process and reactive astrogliosis is also related to neuroinflammation (Davidson et al. 2015). In our study, TH started earlier (TH-2 h) decreased GFAP fluorescence intensity in the CA1 area, suggesting a reduction in reactive astrogliosis for both, males and females.

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Reduction in volume of brain injury and in percentage of degenerative cells are important factors indicative of the efficacy of TH; however, it is well known that brain injury caused by HI can extend for weeks to months (Davidson et al. 2015). Neuroinflammation is one of the main causes that extends the injury for longer periods. It is also known that male animals submitted to neonatal HI show increased microglial activation and upregulation of the inflammatory response (Netto et al. 2017). Astrocytes play a role in this process and reactive astrogliosis is also related to neuroinflammation (Davidson et al. 2015). In our study, TH started earlier (TH-2 h) decreased GFAP fluorescence intensity in the CA1 area, suggesting a reduction in reactive astrogliosis for both, males and females.

In brain injury, an effect that was not seen when hypothermia was started later (6 and 12 h following HI) (Sabir et al. 2012). In addition, Park and colleagues (2015) did not observe neuroprotective effects when hypothermia was started 6 h after injury (Park et al. 2015), corroborating with our findings.

In order to strengthen analysis of brain injury, we decided to calculate the pathological score (Thoresen et al. 1996) of brain structures from which the injury volume was assessed. The correlation between data of pathological score and lesion volume showed a very strong association proving both forms of analysis can be used. Although analysis of volume of brain injury and pathological score demonstrated a beneficial effect of hypothermia initiated 2 h after HI, no sex-related differences were observed in such parameters.

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However, to obtain a more detailed assessment of astrocyte activation, we used Sholl’s concentric circles method to quantify astrocyte complexity (arbour length and number of processes) (Mestriner et al. 2011; Mari et al. 2019). These parameters were evaluated because increase in GFAP immunoreactivity as well as increase in number and compliance of processes are characteristics of reactive astrocytes as already demonstrated in other studies (Haim et al. 2015; Liddelow et al. 2017; Sheikhbahaei et al. 2020). Sholl analyses showed that the TH-2 h female group had a reduced number of primary processes as well as length of processes compared to the HI group, which was not observed in males. Morphology of astrocytes may reflect the functional significance of neuroglial interactions (Sheikhhbahaee et al. 2018). The glial scar formation triggered by neural lesions such as those produced after hypoxic-ischemic events, when uncontrolled, may impair neural communication and cell function (Sofroniew 2015; Sheikhhbahaee et al. 2018). Therefore, although GFAP fluorescence intensity was used to infer increased astrocyte reactivity, females from the TH-2 h group showed smaller number of primary processes and of shorter length, indicating more organized glial scar formation; this is conceivably beneficial for tissue repair (Sizonenko et al. 2008).

Astrocyte reactivity parameters (evaluated by GFAP immunofluorescence intensity and Sholl circles) also showed a strong association with percentage of degenerative cells in CA1. It was expected, since reactive astrogliosis is a phenomenon in which astrocytes branch off and extend their processes to form a glial scar at the locations where neurons have died. A study by Reddy and colleagues (2020) also observed a correlation between volume of hippocampus and the reactive astrogliosis marker GFAP+, corroborating the hypothesis of glial scar formation (Reddy et al. 2020).

The HI model promotes neuroinflammation in two distinct phases: early and late phases. The early phase is characterized by direct activation of microglia leading to the production of pro-inflammatory cytokines, which can also lead to reactive astrogliosis, thus contributing to the late inflammatory phase (Deguchi et al. 1997). Therefore, since microglial activation is known to promote astrocyte activation, the amplification of neurotoxicity can be explained by the increase in glutamate and inflammatory cytokines released by astrocytes (Bezzi and Volterra 2001).

### Table 1  Correlation between histological and behavioural data separated by animals’ sex.

| Males | Volume of Lesion | Behavioral Assessment | Immunofluorescence (GFAP) |
|-------|------------------|-----------------------|---------------------------|
|       | Hemisphere       | Hippocampus           | Cerebral Cortex           |                   |
|       |                  | Negative Geotaxis     | Righting Reflex           | Fluorescence Intensity | Primary processes | Distance from soma |
| Volume of Lesion | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ |
| Hemisphere      |      |      |      |      |      |      |      |      |
| Hippocampus     |      |      |      |      |      |      |      |      |
| Cerebral Cortex |      |      |      |      |      |      |      |      |
| Degenerative Cells |      |      |      |      |      |      |      |      |
| CA1 of Hippocampus |      |      |      |      |      |      |      |      |
| Pathology Score |      |      |      |      |      |      |      |      |
| Hemisphere      | 0.9538** |      |      |      |      |      |      |      |
| Hippocampus     | 0.9028** |      |      |      |      |      |      |      |
| Cerebral Cortex | 0.9066** |      |      |      |      |      |      |      |

| Females | Volume of Lesion | Behavioral Assessment | Immunofluorescence (GFAP) |
|---------|------------------|-----------------------|---------------------------|
|         | Hemisphere       | Hippocampus           | Cerebral Cortex           |                   |
|         |                  | Negative Geotaxis     | Righting Reflex           | Fluorescence Intensity | Primary processes | Distance from soma |
| Volume of Lesion | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ | $r^2$ |
| Hemisphere      |      |      |      |      |      |      |      |
| Hippocampus     |      |      |      |      |      |      |      |
| Cerebral Cortex |      |      |      |      |      |      |      |
| Degenerative Cells |      |      |      |      |      |      |      |
| CA1 of Hippocampus |      |      |      |      |      |      |      |
| Pathology Score |      |      |      |      |      |      |      |
| Hemisphere      | 0.9258** |      |      |      |      |      |      |
| Hippocampus     | 0.9237** |      |      |      |      |      |      |
| Cerebral Cortex | 0.9264** |      |      |      |      |      |      |
In addition, it has already been observed that male animals have increased microglial activation and upregulation of the peripheral inflammatory response compared to females 3 days after being subjected to the HI model (Mirza et al. 2015; et al., 2015), which may be one of the explanations to observe in our study why females show a reduction in reactive astrogliosis.

In addition to the morphological parameters, an analysis of neurodevelopmental parameters is also important and can provide broader data on the neuroprotective effects of TH. Previous studies from our group (Tassinari et al. 2020) and from others (Lubics et al. 2005) have shown that HI causes an increase in latency to complete the negative geotaxis test, as seen here. However, together with the neuroprotective effects of TH on morphological and structural variables, we have also observed an improvement in the behavioral response in the test of negative geotaxis in animals from the TH-2 h group, for both sexes. Jatana and colleagues (2006) showed that hypothermia was not able to reduce the latency in the negative geotaxis test 7 days after injury; however, the hypothermia protocol lasted for only 2 h, which could explain differences in the results (Jatana et al. 2006). By starting hypothermia 3 h after injury and using a prolonged period of hypothermia (48 h), Ahn and colleagues (2018) observed a reduction in latency to perform the negative geotaxis test when animals reached 42 days of life (Ahn et al. 2018), but not at P14, P21, P28, and P35. It has already been observed in animal models that a protocol of hypothermia longer than 5 h produces no additional neuroprotective effects (Sabir et al. 2012). Moreover, prolonged periods of maternal separation produce negative effects on behavior (Lehmann et al. 1999), which could explain the reason why longer periods of hypothermia do not lead to improvement in behavioral outcomes.

When the righting reflex was evaluated, only males from the TH-2 h group showed a reduction in latency to complete the test as compared to the HI group. Yuan et al. (2015) also observed that hypothermia tended to reduce the latency to complete the righting reflex test compared to the HI group; however, sex-dependent effects were not assessed (Yuan et al. 2015). In the present study, TH was not able to improve the female response in the righting reflex test, regardless of the period in which it was started (2 h, 4 or 6 h after HI). Behavioral improvements observed in males and females in the negative geotaxis test, as well as in males in the righting reflex test, may be related to the reduction in volume of lesion and decreased percentage of degenerative cells observed in these groups. To test this hypothesis, a correlation between behavioral performance, morphological and structural parameters was run. We observed only a weak to moderate association between these variables, depending on the parameters evaluated.

Studies on sex dimorphism is still scarce in the neonatal HI model, and even less present when it comes to the experimental effects of hypothermia following HI. However, there is evidence of differences between females and males in other behavioral tests using animals submitted to the neonatal hypoxia-ischemia model, these studies suggest a worse performance than males during the training period for spatial tasks. In general, males subjected to hypoxia-ischemia spend more time in the platform area consistently with task acquisition during training (Sanchez et al. 2013b; Waddell et al. 2016). As regards to training, there are studies reporting no sexual dimorphism of spatial memory function (Arteni et al. 2010; Peterson et al. 2015).

The hippocampus is a structure that play important roles in spatial navigation and episodic memory (Soltesz and Losonczy 2018). Studies using gamma radiation showed alterations in hippocampal layers with pyramidal neurons exhibiting degeneration, i.e., showing characteristics of pyknosis, karyorrhexis and karyolysis (Li et al. 2014; Owoeye and Malomo 2015). Death of pyramidal neurons can disrupt the flow of information suggesting that memory and other functions of the hippocampus could potentially be affected (Owoeye and Malomo 2015).

Furthermore, among the structures evaluated in the present study, the hilus has a regulatory role for neuronal migration in neonates, which can continue into the adult stage (Saegusa et al. 2010, 2012). Lesions in this area may suggest alterations in development and plasticity of the hippocampus, which may reflect behavioral changes. However, behavioral tests used here are characterized by reflex movements and are more related to brain areas not evaluated in this study such as the brainstem (Heyser 2003; Schneider and Przewlocki 2005).

Our study has some limitations. First, we evaluated animals using only one time point (P14, i.e., 7 days after the HI event) and it is well known that the brain lesion can mature from days to months (Davidson et al. 2015). Therefore, further studies using animals from both sexes treated with TH and tracked for longer periods are needed, which would allow assessment of cognitive function using specific behavioral tests in adulthood. Second, we did not evaluate molecular mechanisms underlying the neuroprotective effects of TH. It is well described that TH has an effect in reducing brain metabolism leading to delayed cellular depolarization and, thus, reduction of intracellular calcium influx; however, understanding the molecular basis of TH effects can increase knowledge of its functioning and allow the combination of TH with other adjuvant therapies, in order to increase neuroprotective benefits (Davidson et al. 2015; Cho et al. 2020).
Conclusions

Summarizing, we have shown that TH started early after HI injury led to reduction in brain damage and reactive astrogliosis, as well as an improvement in the neurological reflexes deficits caused by HI, for both sexes. However, when TH was initiated later (i.e., 6 h after HI), females showed an increase in percentage of degenerative cells in the hippocampus ipsilateral to the lesion. Females from the TH-6 h group also showed an increase in latency to perform behavioral tests.

To the best of our knowledge, this is the first report showing that the effects of TH on reactive astrogliosis are dependent on animals’ sex. Therefore, we emphasize the importance of starting TH as soon as possible for both sexes. If there is a need for the treatment to be started later, even greater care should be given to females, as a later start of TH treatment can intensify brain injury in females.

Author contributions All authors contributed in this work. RBF, LSF, CAN and planned the study design. RBF, RRN, MMM, MKGA, APRM, IDT and ESF conducted the experiments. RBF performed data analysis and wrote the manuscript. RBF and IDT prepared the figures. RBF, EFS, LFS and CAN revised critically the manuscript. LSF and CAN were recipient of funding used to support the study.

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Data Availability “The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request”.

Declarations

Competing interests The authors declare that they have no conflict of interest.

Ethics approval All procedures involving animals were in accordance with the precepts of the National and International Guidelines, especially Law 11,794 of November 8, 2008, Decree 6899 of July 15, 2009, and as edited by the National Council for the Control of Animal Experimentation (CONCEA), was approved by the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (UFRGS, #31442).

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