# Effects of treatment with inter-alpha inhibitor proteins and therapeutic hypothermia on hypoxic-ischemic brain injury in the behavior of neonatal rats

By

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Thesis

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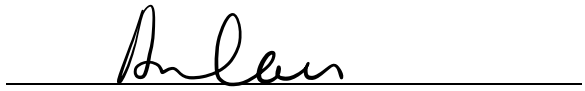
Date 05/01/2023 

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## Preface

The experiments and results of this thesis were completed as a graduate student research assistant in the Perinatal Brain Research Laboratory at the Kilguss Research Institute, the Women & Infants Hospital of Rhode Island from Sep. 2021 to April 2023. The IAIP for injection was provided by Yow-Pin Lim, MD, PhD. Parts of the experiments including the surgeries and injections are supported by Xiaodi Chen and Yuqi Wu. The model of Hypoxia-Ischemia used in study was developed by Dr. John E. Rice, M.D., and Dr. Robert C. Vannucci at the Department of Pediatrics, the Milton S. Hershey Medical Center at Pennsylvania State University.

**Acknowledgement**

Xiaodi Chen – Thank you so much for your invaluable support, guidance, and patience for the research. Your passion and expertise in the research encouraged me to work hard. I feel very proud to be part of this project and I have learned so much from you. Time went fast but the memories would stay with me forever. Best wishes to you and the lab.

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## Introduction

*HIE in neonatal brain*

Hypoxic-ischemic encephalopathy (HIE) is a type of neuronal dysfunction. It occurs when the brain has limited oxygen or blood flow. Infants may experience HIE before birth, during the delivery, or after the birth. The severity of brain damage depends on how long the brain experiences the lack of oxygen and blood flow. The consequences of HIE range from no significant following impact to severe disabilities. Examples are developmental delay, cognitive impairment, cerebral palsy (a disorder that affects the ability to move and maintain balance and posture) or epilepsy (Allen & Brandon, 2011). About 1.5 per 1,000 newborns are identified with HIE with different severities in developed countries and 23 percent of neonatal deaths worldwide (Allen & Brandon, 2011). In developing countries with limited resources for HIE treatment, the prevalence and mortality are even higher. In some developing countries, the number of affected cases increases to 26 out of 1,000. (Black et al., 2010)

By the age of 2 years, up to 60 percent of the infants with HIE will die or have severe disabilities including mental retardation, epilepsy, and cerebral palsy(Allen & Brandon, 2011). There are no effective ways to prevent HIE happening even with advanced neonatal care and monitoring since the incident has not declined even with advances in obstetric care that aimed at preventing the hypoxic-ischemic event. (Allen & Brandon, 2011) Because of the high mortality and the long-term cognitive effects, Hypoxic-ischemic encephalopathy becomes the priority issue for the healthcare system to prevent in neonatal infants.

HIE could occur at any point during pregnancy, delivery, and after birth. The causes of HIE during pregnancy could be issues with blood flow to the placenta, high blood pressure and heart disease of the mom, problems with the formation of lungs, congenital infections, and anemia in the fetus. Causes during the delivery period could be bleeding from placenta, low blood pressure in the mom, umbilical cord problems, and the rupture of the uterus. Causes in the postnatal period could be severe lung or heart disease, infections, respiratory failure or cardiac arrest, and low blood pressure. While sometimes the exact causes are not always identified, cord prolapse, uterine rupture, abruptio placenta, placenta previa, maternal hypotension, breech presentation, or shoulder dystonia are common causes of HIE.(Allen & Brandon, 2011)

*Effects of HIE on the behavior*

Among the long-term effects of HIE on patients, cognitive impairments are considered one of the most critical concerns because it may cause long term behavior deficits and academic performance. Neuronal and cognitive development could be impaired by HIE with or without the motor function deficits in survivors (Lee & Glass, 2021). However, to what extent the HIE would cause the behavioral difficulties is a more complicated case, since some cognitive functions are not fully developed during this early stage (Lee & Glass, 2021). There is a strong correlation between the severity of the HIE and the impairment of the cognitive and motor function. In general cases, infants suffering from severe HIE would be more likely to have impaired neurodevelopment. On the other hand, infants with mild to moderate HIE would likely have normal cognitive outcomes in their early life stages (Lee & Glass, 2021).

*Pathophysiological response in HI injury*

The pathophysiology of HIE could sometimes be complicated and hard to identify. However, the most common underlying event for the HIE development is the impaired cerebral blood flow and oxygen delivery to the brain. The pathophysiology of HIE could be divided into four phases, hypoxia-ischemia phase, latent phase, secondary phase, and tertiary phase. (Figure 1.)

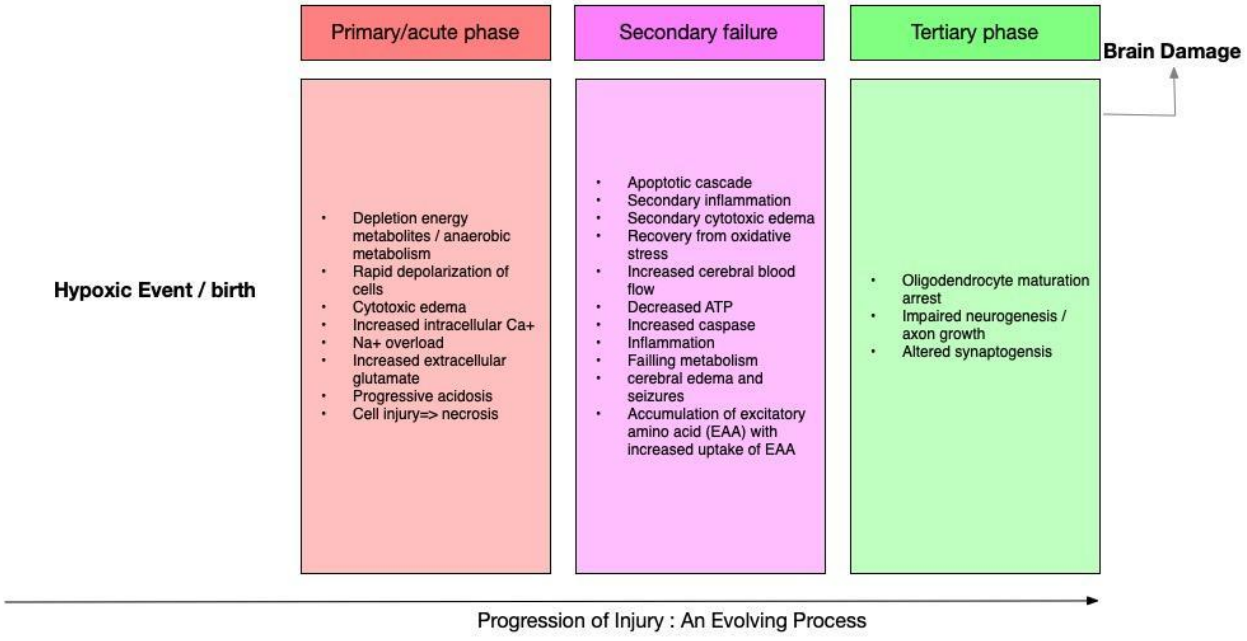


*Figure 1. Phases in hypoxic-ischemic brain injury*

The first acute phase usually occurs within minutes. The impairment of cerebral blood flow leads to decreased oxygen and glucose.(Allen & Brandon, 2011) The reduction of oxygen and blood supply followed by the decrease of ATP, increased lactate production, failure of the sodium and potassium pump, depolarization of cells and in some severe cases, the necrosis of cells. Necrosis occurs in very severe conditions. It causes the cells to swell and easily ruptured, leading to the cell death. The cellular contents that are released by the ruptured cells would cause further inflammation(Allen & Brandon, 2011) When the sodium/potassium pump fails, a large influx of

the positively charged sodium ions will lead to the depolarization of neurons. After the resuscitation and repercussions, the latent phase could last up to 15 hours. During the latent phase, the impairment of cerebral oxidative metabolism could partially recover before the irreversible failure of the mitochondria function.

The secondary phase is where the most neonatal brain damage occurs. This phase usually occurs 6 to 48 hours after the initial injury. Massive influx of calcium, oxidative stress, more inflammatory cytokines are released and finally the programmed cell death. The increased intracellular calcium level is detrimental since it leads to cerebral edema, ischemia, and microvascular damages.(Allen & Brandon, 2011) The process of this phase could last for days before entering the tertiary phase. The final phase is when the brain damage persists and lasts for months to years after the HI injury. (Yıldız et al., 2017)

The energy failure phase in the early stages of HIE initiates a series of detrimental biochemical cascades. Free oxygen radicals produced from xanthine byproducts and prostaglandin synthesis attack polyunsaturated fatty acids of the plasma membrane, resulting in the increased plasma membrane permeability. (Leonardo & Pennypacker, 2009)  The electron transport chain in mitochondria during cell respiration is also one of the major players generating reactive oxygen species. Thus, the mitochondrial dysfunction contributes to the necrosis of injury cells as well. (Leonardo & Pennypacker, 2009) The dysfunction of ATP dependent ion pumps causes cellular depolarization and glutamate release into the extracellular space.(Leonardo & Pennypacker, 2009) Considering all of the chemical cascade mentioned above, the responses of HIE in the

early stages are the neuronal necrosis resulting from excitotoxic damage. (Leonardo & Pennypacker, 2009)

More cell deaths could occur after the early responses of HI injury caused by the immune response in the brain. The release of proinflammatory cytokines is responsible for the neuroinflammation during the secondary phase. Accumulating evidence suggests that targeting delayed neuroinflammatory mechanisms may be a promising neuroprotective strategy to attenuate HI related injury in the developing brain. (Leonardo & Pennypacker, 2009)

*Therapeutic hypothermia*

Currently the only approved therapeutic treatment for HIE in full-term infants is hypothermia, also referring to the cooling method. Moderate to severe HIE in full term infants could be treated with hypothermia by decreasing and maintaining the body temperature of 33.5 degree Celsius for 72 hours within the first 6 hours of life. (Karnatovskaia et al., 2014) Hypothermia is also the first therapy to show the neuro-protective effects in newborn animals. In large randomized clinical studies, the mortality rate in newborns treated with hypothermia significantly decreased with improved neurodevelopmental outcomes. (Karnatovskaia et al., 2014) The mechanism of hypothermia as protective treatment for HIE is that it provides the environment that inhibit the activity of a variety of negative cell activities and chemical cascades. The positive effects of hypothermia include decreasing apoptosis, retain the loss of high-energy phosphates, reduced oxygen consumption, reduce the release of nitric oxide, glutamate, free radicals and excitatory amino acid neurotransmitters, and the induction of genes that could regulate the apoptosis and necrosis. (Karnatovskaia et al., 2014)

However, the therapeutic window for hypothermia is limited to the latent phase. Studies showing that extending longer periods of hypothermia or lower cooling temperatures in full term infants would hardly result in further beneficial effects.(Shankaran et al., 2014) Therapeutic hypothermia could only be applied to full-term but not preterm infants, which means there are still a large number of newborns who would not have effective therapies for HIE. Hypothermia would only be effective in cases with mild to moderate HIE, but in severe cases, it would not be effective in reducing major neurodevelopmental disabilities and mortality when performing alone. (Tagin et al., 2012) The use of therapeutic hypothermia in preterm infants may result in higher mortality rates and potentially developing more complicated diseases.(Rao et al., 2017)

The prevalence of HIE in developing countries is in fact higher than developed countries, where they have limited resources and techniques to get access to the treatments and even identify the symptoms from the early stages of HIE. Intensive care and monitoring are needed when implementing the cooling therapy, which countries with low average income and limited resources would not have the facilities to perform, so that the care of the newborns are not in the optimal condition.(Pauliah et al., 2013) Since the optimal therapeutic time for hypothermia is within 6 hours of birth, the beneficial effects of hypothermia might decreased with the delayed admission of hospital or the prolonged labor during the birth. Overall, the feasibility and range of application of performing therapeutic hypothermia in newborns is limited as an effective treatment especially in low-income countries.

Therefore, the need for developing a novel treatment that could provide a neuroprotective effect with larger therapeutic window and broader range of application is the priority of the health providers. This novel agent could act as an alternative or act together with therapeutic hypothermia to extend the therapeutic window and to restore or protect neuronal damage.

*Potential alternative HIE treatments*

Since therapeutic hypothermia could not be applied as an effective treatment to all patients, The development of alternative or adjunct therapy are highly in need. An optimal biomarker should be able to provide sensitive results with more efficiency and help physicians to identify the severity of HIE in order to provide more accurate and timely treatments. Erythropoietin is a type of neuroprotective agent that is hypothesized to be a good candidate. It has some supportive preclinical evidence but a recent study showed that the use of EPO results in higher incidence of death with more neuronal impairments, and severe side effects (Wu et al., 2022). Xenon is another neuroprotective agent, but the optimal dosage and therapeutic window is not determined yet, and it is awaiting more data from clinical trials. Also, it is high-cost and specialist administration is required (Thoresen et al., 2009). Our lab suggested that agents that could attenuate neuroinflammation would be an effective approach to help evaluate the therapeutic efficacy and responses in real time.

*Inter-Alpha Inhibitor Protein*

Inter-alpha Inhibitor Protein are a family of serine protease inhibitors that are found in human and rodent plasma. It is present in high concentrations in the plasma of both preterm and full-term infants. They are naturally occurring novel anti-inflammatory immunomodulatory

proteins that inhibit the serine protease and the release of inflammatory cytokines. (Koehn et al., 2022) The major forms of the Inter-alpha Inhibitor protein found in human plasma are the Inter-alpha Inhibitor (IaI) and pre-alpha inhibitor (PaI). Inter-alpha Inhibitor (IaI) contains two heavy chains and a single light chain, and the pre-alpha inhibitor (PaI) has one heavy chain and one light chain. (Potempa et al., 1989) The light chain, also known as the bikunin, includes a serine protease inhibitor domain that could inhibit the serine protease activities. (Potempa et al., 1989) The primary function of the heavy chain is to stabilize the extracellular matrix and coordinate the activities of bikunin.(Zhuo & Kimata, 2008)

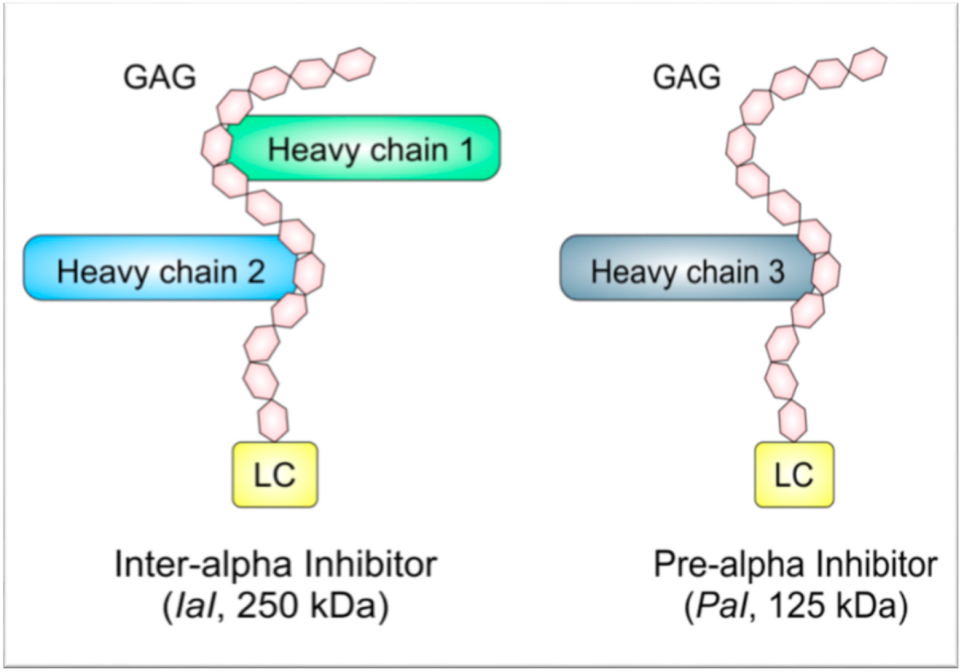


*Figure 2. (Koehn et al., 2020) The structure of major IAIPs present in human serum, IaI and PaI. IAIPs contain a glycosaminoglycan (GAG) backbone of chondroitin sulfate disaccharide repeats, connecting to protein components and termed heavy chains(Koehn et al., 2020). Both IaI and PaI have a light chain attached but connected with different heavy chains.*

*Neuroprotection function of IAIP*

Hypoxic-ischemic encephalopathy could increase the release of proinflammatory cytokines and the activation of matrix metalloproteinases, which can result in further breakdown of the blood brain barrier in the brain and the infiltration of peripheral immune cells. (Chen et al., 2018) Previous works suggested that the light chain, the bikunin, has neuroprotective effects.(Shu et al., 2011) The inflammatory response of neurons contributes to the development of perinatal brain injury during the limited oxygen and blood flow stage and IAIP exhibit the anti-inflammatory and neuroprotective effects in brain injury.(Singh et al., 2010) Furthermore, the treatment with repetitive doses of IAIP (30-60 mg/kg) retain the brain weight loss and alleviate injuries within the cerebral cortex, hippocampus, caudate/putamen, cingulate, and thalamus.(Chen et al., 2019) We showed that treatment with blood-derived IAIPs attenuate the neuronal cell death and reduces the infarct volume in the neonatal brain by 30-35% in both male and female rats. (Chen et al., 2019) These findings suggest that introducing systematic treatment of IAIP could preserve the brain tissue loss and attenuate brain injuries after exposure to hypoxic-ischemic in neonatal brains.

In addition to brain tissue preservation, treatment with IAIP can also limit the cellular activities induced by hypoxic-ischemic environments. The reduction of oligodendrocytes and neurons is the major effect after exposure to HIE, and recent studies show that systematic treatment with IAIPs can reduce neuronal cell death in the cerebral cortex of neonatal rats after HI injury (Chen et al., 2019; Threlkeld et al., 2014). Another study conducted by our lab shows that treatment with IAIPs reduced the neuronal cell death in the cortex and total hemisphere and increased total area of oligodendrocytes by CNPase in the ipsilateral hemisphere and corpus callosum of

neonatal male rats (Chen et al., 2019). Besides the neuronal and oligodendrocytes loss, increased immune cells contribute to the neuroinflammatory responses significantly. Microglia, astrocytes, and leukocytes get increased when exposure to HI, and studies show that treatment with IAIP modulates the activities of these neuroinflammatory immune cells (Barrios-Anderson et al., 2019).

*Neurobehavioral Effects after systematic treatment of IAIP*

The impairment of brain tissues and neuron cell death are closely associated with long-term cognitive ability. Studies show that groups exposed to 8% oxygen for 2 hours then treated with IAIP performed better in water maze tasks (Threlkeld et al., 2014). Additionally, in a recently published small open field study, rats were exposed to HI but without the treatment of IAIP and therapeutic hypothermia have lower area of exploration compared with rats exposed to HI but treated with IAIPs (Koehn et al., 2022). Furthermore, adult rats treated with IAIP after exposure to HI exhibit better auditory discrimination capacity compared with placebo groups (Threlkeld et al., 2014) . These findings suggested that IAIPs act as the neuroprotective agent that could retain both the short- and long-term cognitive functions.

## Objective

Hypothesis: Inter-alpha inhibitor Protein as the alternative and adjunct treatment with therapeutic hypothermia can restore cognitive behaviors and motor deficits in neonatal rats.

The hypothesis was based on the previous study in our lab Inter-alpha Inhibitor Proteins treatment alone improved the righting reflex and Morris water maze behavior of neonatal rats after exposure to HI (Chen et al., 2022 ; Koehn et al., 2022).

## Methods and Materials

*Animal preparation*

The animals were approved by the Warren Alpert Medical School of Brown University and the Women and Infant Hospital to use in experiments described in this paper. The procedure of animal handling and preparation followed the National Institute of Health guidelines of the use of experimental animals. Pregnant Wistar rats on embryonic day 15 or day 16 were purchased from the Charles River Laboratories and housed in a 12-hour light/dark cycle monitor with access to food and water in Animal Care Facilities at Brown University. The birth dates of the rats were designated as P0. There are approximately equal numbers of males and females rats at P1 to limit the inter-litter variability. The pups were randomly assigned within each litter, so that it is possible to have different numbers of males and females pups in each litter even with the attempt to equalize the sexes within the litters.

*Preparation of IAIPs*

Pure (>90%) and biologically active IAIPs were extracted from fresh frozen human plasma (Rhode Island Blood Center, RI, USA) through a series of purification procedures (Toyoperal GigaCap Q659, Tosoh Bioscience, King of Prussia, PA, USA and Astrea Bioseparations, Cambridge, UK). The purity and the activity of IAIPs were determined by the sodium dodecyl sulfate polyacrylamide gel electrophoresis, western immunoblot, protein assay and competitive immunoassay (Threlkeld et al., 2014 ; Lim et al., 2005). Endotoxin in the purified products was evaluated through a Limulus amebocyte lysate endotoxin-based chromogenic analysis (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA).

*Surgery and study design*

The Rice-Vannucci method was used to induce HI, by unilateral carotid artery ligation followed by hypoxic exposure to 90 min of 8% $O_2$ at P7 (postnatal day 7). After recovery from HI, the pups were treated either with normothermia (rectal temp 36°C) or hypothermia (30°C) for 3 h. The rats were randomly assigned to 6 groups:

- Sham +Normothermia (n=25)
- HI + Normothermia + PBS (Placebo) (n=41)
- HI + Normothermia + IAIP (n=26)
- Sham + Hypothermia (n=24)
- HI + Hypothermia + PBS (Placebo) (n=41)
- HI + Hypothermia +IAIP (n=26)

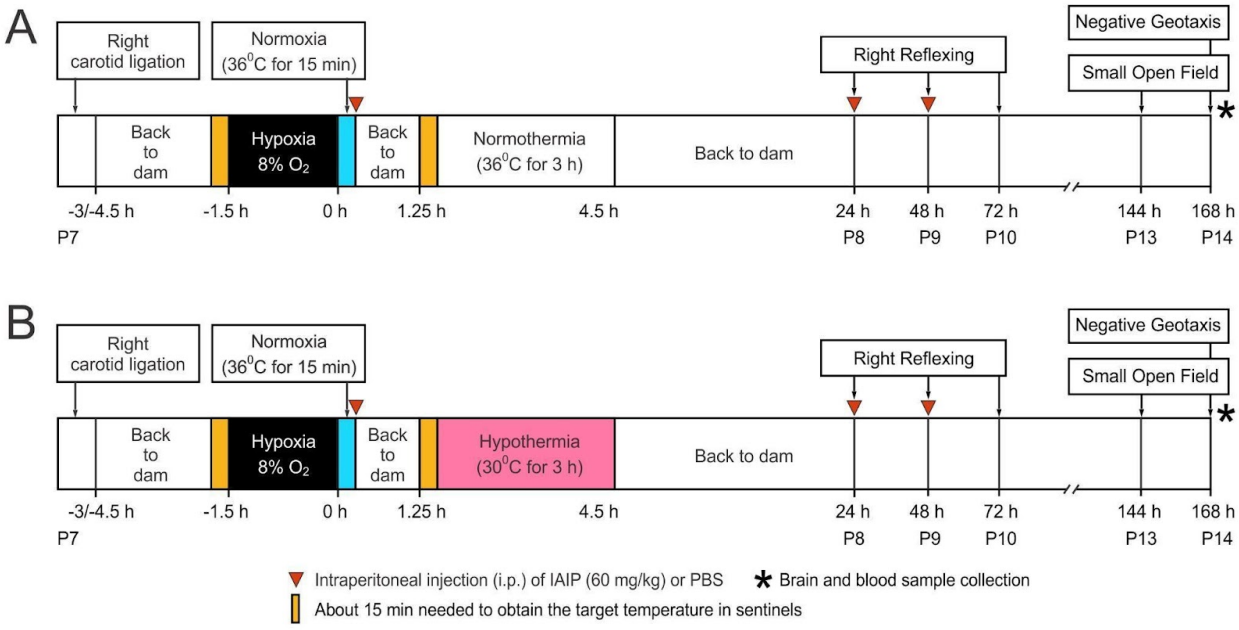


*Figure 3. Hypothermia and IAIP injection study design after postnatal day 7.*

HI animals received PL (phosphate buffered saline) or IAIPs (60 mg/kg) i.p. at 0-0.25, 24, and 48 h after HI. The first dose of IAIP was injected at zero to 15 min after HI. Our previous studies showed that the 30 mg/kg dose of IAIPs improved pathological brain injury, infarct volume, and limited neuroinflammation in neonatal rats 3 days after exposure to HI (Chen et al., 2019). The second dose of IAIP was injected 24 hours after the exposure. The third dose of IAIP was given 48 hours after the exposure of HI. We performed multiple doses injection of IAIP because we believe that maintaining the level of IAIPs over time would have a sustained effect throughout the studies. The pups in HI + Normothermia + PBS (Placebo) and HI + Hypothermia + PBS (Placebo) were given equal volumes of phosphate buffered saline (PBS) at the same time point and intervals through intraperitoneal injection.

*Righting reflex*

The righting reflex study was performed on the postnatal day 8, 9, and 10 (24, 48, and 72 hours after exposure to HI or sham treatment) (Chen et al., 2022). The pups were placed in supine position on a flat surface and were given a maximum of 20 seconds to right themselves to a prone position with all of their paws flat on the surface (Koehn et al., 2022). The time to right themselves was recorded when the pup had placed all four paws flat on the surface. All experiments were video-recorded and analyzed by two independent observers that were blinded for the group designations of each trial.

*Negative geotaxis behavior study*

Negative geotaxis performance was examined on P14, 168 h after exposure to HI or sham treatment. The pups were placed in the center of a 30° inclined ramp (26 × 20 cm), facing downwards (Figure 2) (Koehn et al., 2022). This study is to measure how well the rats can adjust their position after being released from the starting point, and time that took to climb up the hill. Rats with or without brain damage were randomly assigned to groups and were placed on the starting point of the ramp individually. A timer will start at the time when the rats were placed at the starting point facing the direction down the hill and will stop when the rats reach the top of the hill. The experimenter removed their hands once four paws of the pups were flat on the surface when they could support their own weight without the aid from the experimenter. The time taken for the pups to turnover and face the top of the incline were recorded. The time taken for the pumps to reach the top of the incline once they turned over was recorded as well. Each pup completed the task three times. All experiments were video-recorded and analyzed using EthoVision XT tracking software.

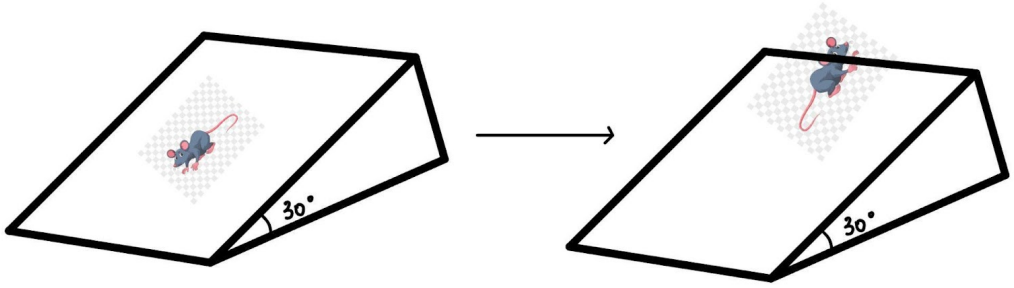


*Figure 4. Field design of negative geotaxis.*

*Small open field*

Small open-field tasks were conducted on P13 and P14, 144 and 168 h after exposure to HI and sham. Pups were placed in the center of an open-field box (28 × 28 cm) and allowed to explore

for 10 min (Koehn et al., 2022). All experiments were video-recorded and analyzed using EthoVision XT tracking software. The arena was segmented into 16 even squares for analysis of zone entry and time spent in the edge and center zones of the arena (Figure 3). The endpoints analyzed were distance traveled, velocity, time in the outer 12 segments, time in the center 4 segments, number of zones entered, acceleration, average time moving, activity, and body elongation state (contracted, normal, and stretched).


*Figure 5. Small open field arena settings.*

*Data collection and Noldus EthoVision XT16*

After the behavior study was video recorded, videos of negative geotaxis and small open fields were imported to the Noldus EthoVision XT16 to track and analyze the behavior, movement, and activity of the animals. (*EthoVision XT - Video Tracking Software | Noldus*, n.d.). The system was set according to the features of the field for each behavior study.

For the negative geotaxis study, all the activities on the ramp were recorded. The time that the rat took to turnover facing up the hill was measured, as well as the time the rat took to climb up the hill (Chen et al., 2022; Koehn et al., 2022). Beside these two measurements, the activities and the total distance moved as it reached the destination were recorded and analyzed as well. After getting the results for the measurements mentioned above. The time that the rat spent wandering near the starting point with one body length as the diameter, designated as the 'starting zone', was measured. The time that the rat spent in the starting zone could potentially indicate how well it can adapt to the topography of the ramp and how long for it to identify the directions, whether down or up the hill.

For the data collection of the small open field study, the measurements focused on the body elongation, activity, movements, velocity, and total distance moved in a given amount of time (Koehn et al., 2022). The field was divided evenly into a total of 16 zones and the time that the rats spent on the edge or the center of the field and the number of zones getting explored would also be analyzed (Koehn et al., 2022).

*Statistical analysis*

Statistical analysis models were selected based on the data analyzed. After obtaining the data from the EthoVision XT16 tracking software, outliers were identified and eliminated using the ROUT test (GraphPad Prism, San Diego, CA, USA). Then the Shapiro–Wilk normality test was performed to test the distribution of the data. Non-normally distributed data were analyzed by the Kruskal–Wallis test following Dunn's test, whereas normally distributed data were analyzed by one-way ANOVA tests following the Tukey HSD test (Chen et al., 2022; Koehn et al., 2022). All statistical tests were performed using the SigmaPlot 14.5 (Systat Software Inc. Palo Alto, CA, USA), with the exception of the outlier tests (GraphPad Software Inc.). Adjusted p values under 0.05 were considered statistically significant for all the analyses.

## Results

*Righting reflex*

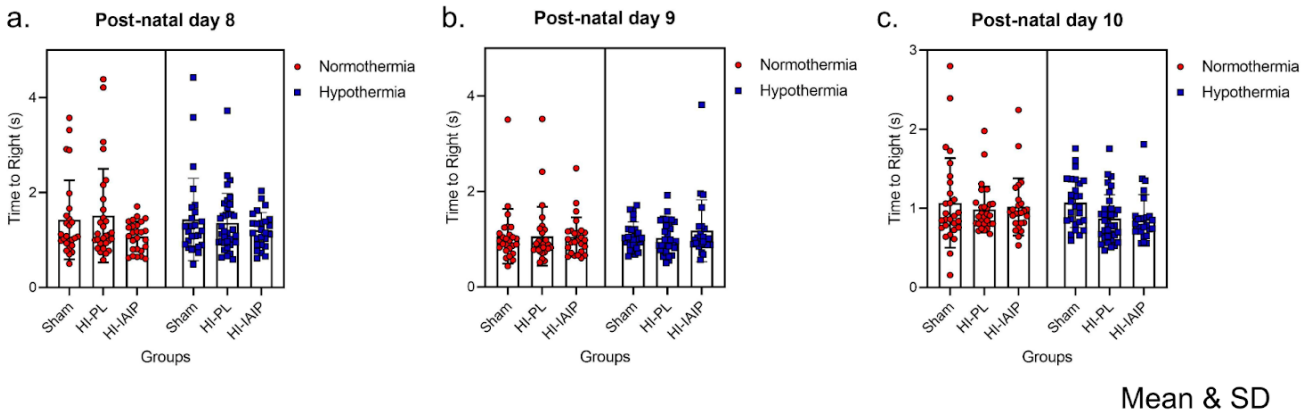


*Figure 6. Righting reflex performance on postnatal day 8 (a), 9 (b), 10 (c), 24, 48, and 72 hours after HI treatment with the first dose of human inter-alpha inhibitor proteins (hIAIPs). Groups are sham normothermia, HI placebo, HI 60 mg/kg hIAIP treated, sham hypothermia, HI placebo with hypothermia, HI 60 mg/kg hIAIP treated with hypothermia. Each animal is presented as an individual datapoint.*

The time to complete the righting reflex tasks for pups at P8, P9, P10 is shown in Figure 5. The time taken to complete the task was not significantly different between any treated group and compared to the sham.

*Negative geotaxis*

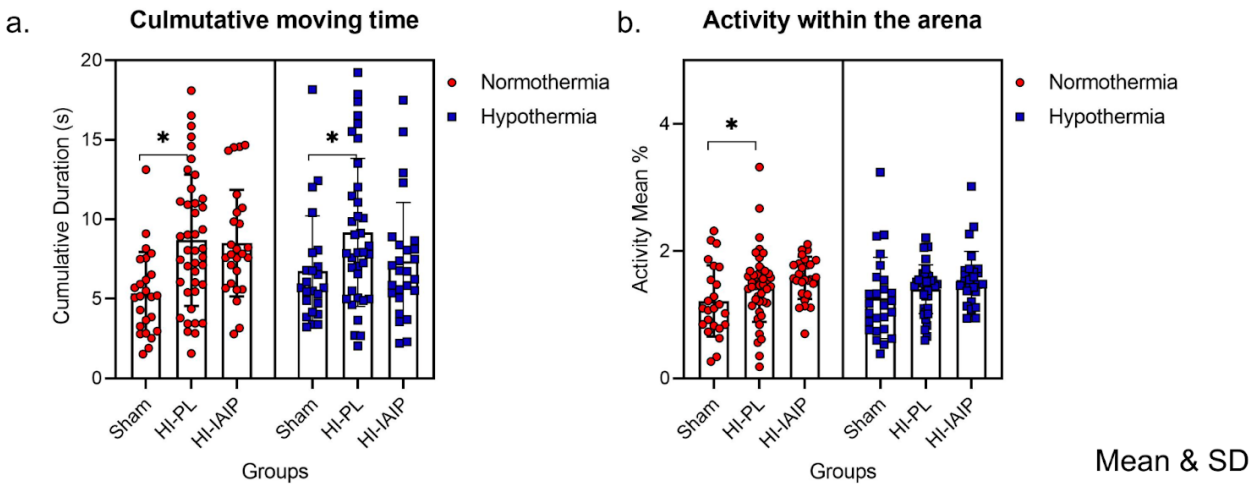


*Figure 7. Negative geotaxis tasks at postnatal day 14 (P14), 168 hours after HI and treatment with the first dose of human plasma-derived inter-alpha inhibitor proteins (hIAIPs). Groups are sham normothermia, HI placebo, HI 60 mg/kg hIAIP treated, sham hypothermia, HI placebo with hypothermia, HI 60 mg/kg hIAIP treated with hypothermia. The cumulative duration of the pups spent on completing the negative geotaxis task on the 30° inclined ramp (26 × 20 cm) (A). The average activity percentage on the 30° inclined ramp (26 × 20 cm) (B). Each animal is presented as an individual datapoint. Statistical analysis was completed using KruskalWallis test, * $p < 0.05$ with Dunn's test for multiple comparisons * $p < 0.05$.*

From the negative geotaxis study, the cumulative moving time in the group treated with hypothermia and IAIP is significantly lower than the group treated with normothermia and place with exposure to hypoxic ischemia. The difference was not statistically significant between the group treated with IAIP alone without hypothermia. The activities of the rats with exposure to hypoxic ischemia but without hypothermia and IAIP treatment was significantly higher compared with the sham group. However, there is no significant difference between groups treated with IAIP or/and hypothermia and groups with exposure to HI but without any treatments.

Differences are not significant between any groups on the time taken to turn 180° facing the top of the inclined plane. These non-significances indicate that this turnover test was not sensitive enough to determine the behavior differences after neonatal hypoxic-ischemic injury in male and female neonatal rats in this study.

*Small open field study*

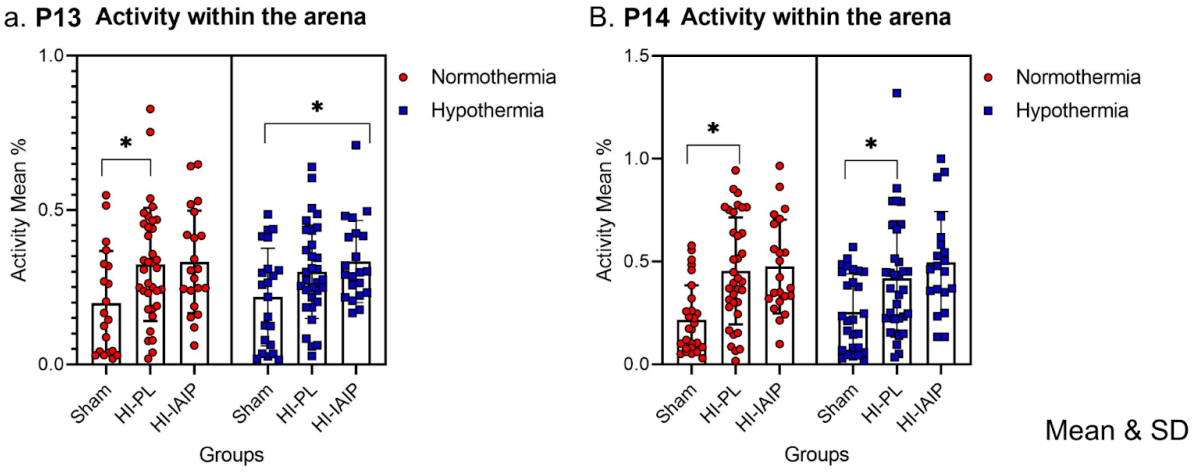


*Figure 8. Small open field tasks at postnatal day 13 (P13), 144 hours after HI and treatment with the first dose of human inter-alpha inhibitor proteins (hIAIPs) (a). Small open field tasks at postnatal day 14 (P14), 168 hours after HI and treatment with the first dose of human inter-alpha inhibitor proteins (hIAIPs) (a). Groups are sham normothermia, HI placebo, HI 60 mg/kg hIAIP treated, sham hypothermia, HI placebo with hypothermia, HI 60 mg/kg hIAIP treated with hypothermia. The average activity percentage exhibited during the 10 minutes exploration period in the 28cm x 28cm open field. Each animal is presented as an individual datapoint. Statistical analysis was completed using Kruskal Wallis test, * $p < 0.05$ with Dunn's test for multiple comparisons * $p < 0.05$.*

An increase in the activities was detected in the HI placebo group compared with the sham in at P13 and P14. The percentage of activity decreased in HI hypothermia, HI hIAIP, and HI hypothermia and hIAIP groups were not significant, therefore this task could not distinguish the differences between the HI placebo, HI hypothermia, HI hIAIP, and HI hypothermia and hIAIP groups of P13 and P14 neonatal rats from this measurement.

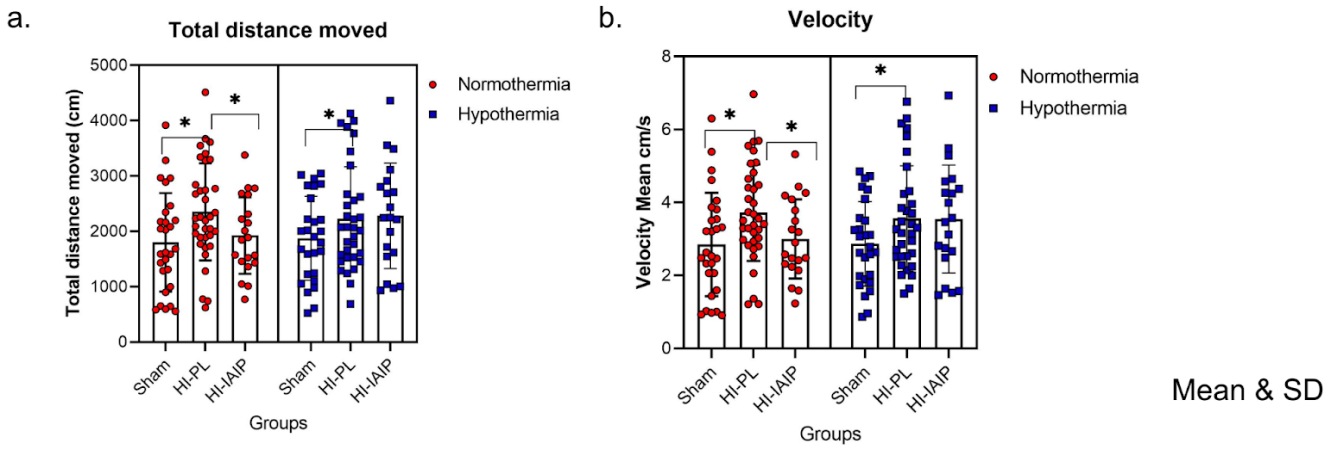


*Figure 9. Small open field tasks at postnatal day 14 (P14), 168 hours after HI and treatment with the first dose of human inter-alpha inhibitor proteins (hIAIPs). Groups are sham normothermia, HI placebo, HI 60 mg/kg hIAIP treated, sham hypothermia, HI placebo with hypothermia, HI 60 mg/kg hIAIP treated with hypothermia. The total distance moved in centimeters during the 10 minutes exploration period in the 28cm x 28cm open field. The average velocity in centimeters per seconds during the 10 minutes exploration period in the 28cm x 28cm open field. Each animal is presented as an individual datapoint. Statistical analysis was completed using Kruskal Wallis test, * $p < 0.05$ with Dunn's test for multiple comparisons * $p < 0.05$.*

Exposure to HI significantly increased the total distance moved in HI placebo groups compared with the sham under normothermia and hypothermia conditions of neonatal rats at postnatal day 14. Same results were reflected in the average velocity measurement. The decrease in the total distance moved and the average velocity in HI hypothermia, HI hIAIP, and HI hypothermia and hIAIP groups were not significant, therefore this task could not distinguish the differences between the HI placebo, HI hypothermia, HI hIAIP, and HI hypothermia and hIAIP groups of P13 neonatal rats from this measurement.

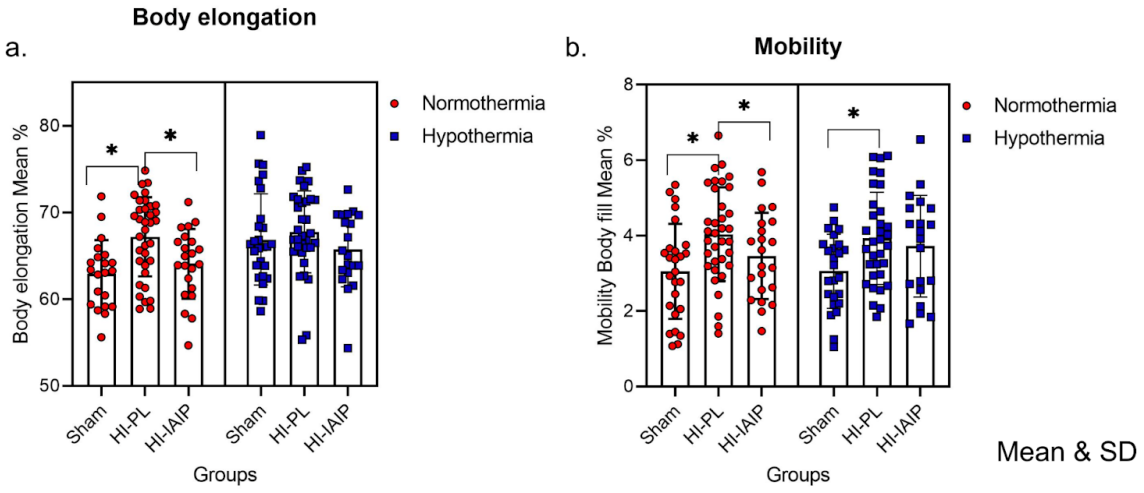


*Figure 10. Small open field tasks at postnatal day 14 (P14), 168 hours after HI and treatment with the first dose of human inter-alpha inhibitor proteins (hIAIPs). Groups are sham normothermia, HI placebo, HI 60 mg/kg hIAIP treated, sham hypothermia, HI placebo with hypothermia, HI 60 mg/kg hIAIP treated with hypothermia. The percentage of extension and contraction postural changes during the 10 minutes exploration period in the 28cm x 28cm open field. The average mobility percentage during the 10 minutes exploration period in the 28cm x 28cm open field. Each animal is presented as an individual datapoint. Statistical analysis was completed using Kruskal Wallis test, * $p < 0.05$ with Dunn's test for multiple comparisons * $p < 0.05$.*

The percentage of time that the body was in the elongated and contracted postures of neonatal rats during the time in the small open field was significantly higher in the HI placebo groups under normothermia and hypothermia conditions. Treatment with 60 mg/kg hIAIP under normothermia significantly reduced the percentage of time that the body was in the elongated and contracted postures compared to HI placebo groups under the normothermia condition. Differences were not significant in the percentage of time that the body was in the elongated and contracted postures between the HI placebo group and HI hIAIP group under the hypothermia condition.

The percentage of the average mobility of neonatal rats during the time in the small open field was significantly higher in the HI placebo groups under normothermia and hypothermia conditions. Treatment with 60 mg/kg hIAIP under normothermia significantly reduced the percentage of the average mobility compared to HI placebo groups under the normothermia condition. Differences were not significant in the percentage of the average mobility between the HI placebo group and HI hIAIP group under the hypothermia condition.

## Discussion

HIE can cause mild to severe brain injury in neonatal infants and can result in irreversible neuronal damages with high mortality rate. The damages of the brain can lead to a large range of neurological developmental diseases and cognitive disorders (Huang & Castillo, 2008). The current approved treatment for attenuating brain injury caused by HIE is therapeutic hypothermia. However, therapeutic hypothermia has a narrow therapeutic window which could only be used in the first few hours after the HI injury. Furthermore, therapeutic hypothermia was only approved as the treatment for full-term infants suffering from the brain injury caused by HIE but was not approved for use in preterm neonates. The range of protection depends on the time of the intervention. However, in counties with low income and limited resources, the timing of the hypothermia intervention and monitoring after the hypothermia intervention could be away from the optimal condition, resulting in partial protection or even no protection. Therefore, the need of developing a novel neuroprotective agent as the treatment for HI related brain injuries for all terms of infants with a wider therapeutic window is urgent for all healthcare providers.

Human inter-alpha inhibitory proteins are novel therapeutic agents that have neuroprotective properties in many preclinical studies (Chen et al., 2022). This study was designed to evaluate the individual and combined effects of therapeutic hypothermia and human inter-alpha inhibitory protein in the cognitive behavior of neonatal rats. The study design of the i.p. injection depends on the half-life and the pharmacokinetics of hIAIP and the pharmacokinetics. Pharmacokinetics analysis from previous studies showed that the concentration of hIAIP reaches the highest

concentration in blood 1-12 hours after the injection, and the half-life of hIAIP is 10-23 hours (Chen et al., 2020).

Systematic treatment of human IAIP has previously been shown to improve the behavior of rats in water maze (Gaudet et al., 2016) and wire hang tasks (Chen et al., 2022). A combination of sensory perception of recognizing themselves are in a supine position, the innate cognitive capacity to recognize that they should be in a prone position, and the motor function to correct to the prone position would be required when performing the righting reflex (Koehn et al., 2022). In this righting reflex study, no significant differences were observed in any of the treatment groups, indicating that this righting reflex test was not sensitive enough to determine the behavior differences between sham and neonates having brain injuries in this study.

Negative geotaxis is a type of automatic unlearned response and direction sensory response against the gravitational cues, developed in the early age of pups as an innate postural response(Ruhela et al., 2019). It is also one of the early behavioral tests that could be used to evaluate motor development, activities, and vestibular functions (Altman & Sudarshan, 1975). As an important early behavior test, negative geotaxis controls the vestibular functions in developmental sequence after the onset of the sensory function (Ruhela et al., 2019). Exposure to hypoxic-ischemia results in a longer cumulative moving time in rats without treatment of hypothermia and IAIP compared with the sham group. Combining the treatment of hypothermia and IAIP significantly reduced the cumulative moving time compared with the HI placebo group (Figure ). The cumulative moving time is an important indicator of the time that the pups used to

climb up the inclined ramp, indicating that rats with brain injury took longer to complete the negative geotaxis task. Since negative geotaxis could be used to evaluate motor development, activities, labyrinthine reflex, strength, and coordination (Diaz et al., 2017) , the result could be the sign of hIAIPs work with hypothermia attenuating the brain injury from exposure to HI.

Neonatal rats exposed to HI exhibit a higher percentage of time with an elongated or contracted body compared with the sham group. This high frequency of postural changes could be the indicator of anxiety or stress (Bailey & Crawley, 2009). Treatment with hIAIP under normothermia in neonatal rats exposed to HI has a decreased percentage of time with elongated or contracted body posture. However, treatment with hIAIP under hypothermia in neonatal rats did not attenuate the frequency of the postural changes. Therefore, we can not conclude that treatment with hIAIP as an adjunct to therapeutic hypothermia could ameliorate the anxiety and distress caused by brain injury from HI exposure.

It was surprising that treatment with hIAIP individually has more improvements in the early stage behavior we measured, and results correspond with the data from previous studies of our lab (Koehn et al., 2022). The suboptimal dosage or the number of doses of hIAIP is potentially responsible for the ineffectiveness of the combined treatment of hIAIP and hypothermia. Previous studies investigating the effect of three doses of hIAIP treatment on neonatal rats suggested that increasing the dosage from 30 mg/kg to 90 mg/kg did not show a further beneficial effect of the behavior and reduction of the infarct volume (Koehn et al., 2022). There might be some undiscovered negative effect of dosage higher than 30 mg/kg of hIAIP as a combined treatment with therapeutic hypothermia. Epo has previously shown to be a

neuroprotective agent that improves the histology function after neonatal stroke. However, studies show that the combined treatment of Epo and hypothermia did not show a beneficial effect (Fang et al., 2013).

The time that the animals were treated with therapeutic hypothermia could also be suboptimal since hypothermia as the only treatment without the injection of hIAIP did not perform well in this study. Magnesium sulfate (MgSO4) is an endogenous anti-excitotoxic agent that was selected as a potential novel therapeutic method to HIE (Zhou et al., 2020). The study investigating the combined treatment effect of MgSO4 and hypothermia also concluded that longer cooling time and delayed introduction of novel neuroprotective agents could possibly improve the performances of the combined treatment (Lingam et al., 2019).

We also measured the total distance moved, velocity, number of zones entered and activities. However, the data were not significant enough to conclude that the combined treatment or individual treatment of hIAIP and therapeutic hypothermia attenuate the effect of HIE on the early cognitive and motor behavior of neonatal rats. The differences might become significant if we extend the duration of the exploration time of the small open field experiment. Increase in the animal numbers of each experimental group would lead to more comprehensive results. More accurate results could be generated by balancing the sex within each litter to exclude the differences in the mechanism of HI-related brain injury between male and female neonatal rats. Investigating the optimal dosage and points of delivery could also be potential approaches to generate more accurate and significant results. Future research could be done to see the

long-term behavioral performances in a later stage after the HI injury with more focus on the gender specific differences.

## Conclusion and perspectives

Systematic treatment of exogenous IAIP is an effective neuroprotective strategy which could attenuate brain weight loss and reduce the infarct volume. Not only the morphological properties could be restored and preserved, studies have shown that IAIP treatment alone can also improve the cognitive, direction, sensory, locomotion, and motor behavior after moderate to severe brain injury in neonatal rodents. More research is required to confirm the adjunctive effects of IAIP treatment to HT from different stages of HIE with more focus on the gender specific differences. Further analysis should also consider the brain function and maturity of the rats when designing the behavior analysis.

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