Hypothermia in preclinical models of neonatal hypoxic-ischemic encephalopathy

Doctoral thesis

Dr. Áron Kerényi

Semmelweis University

Doctoral School of Clinical Medicine

Consulent:	Dr. Miklós Szabó, MD, PhD
Official reviewers:	Dr. Ferenc Domoki, MD, PhD Dr. Krisztián Szigeti, PhD
Head of the Final Examination Committee:	Dr. András Szabó, MD, D.Sc
Members of the Final Examination Committee:	Dr. Tibor Ertl, MD, D.Sc, Dr. Alán Alpár, MD, D.Sc

Budapest

2016

INTRODUCTION

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of the most devastating diseases of the perinatal period. Approximately 1-2 newborns per 1000 live births are affected in the developed countries while its prevalence in the developing world is much higher, altogether accounting for around 700.000 neonatal deaths worldwide annually. Additionally, 1.15 million children are estimated to develop neonatal encephalopathy and almost half a million will suffer lifelong neurodevelopmental impairments due to HIE. Evidently, neonatal HIE presents one of the most severe problems in perinatal care.

Efforts to develop effective therapeutic interventions for children with HIE have been ongoing since the early 19th century. Numerous animal models have been developed in order to shed light on the pathomechanisms and therapeutic opportunities underlying this devastating disease. Large animal models utilizing the fetal or neonatal monkey, lamb and piglet were highly successful in elucidating the various patterns of brain injury as well as the intertwined relationship between hypoxia and ischemia and more recently, via the help of functional imaging modalities, the metabolic processes involved in perinatal asphyxia. At the same time, small animal models have also been used extensively to understand cellular and molecular processes involved in the development of brain injury as well as to test potential neuroprotective therapies. While our understanding of pathomechanisms involved in the development of HIE has grown extensively, especially since the mid-20th century, there has been only one therapeutic intervention, hypothermia, which was found to be clinically safe and effective for children with HIE. There have been more than 500 neuroprotective interventions which showed promise in certain stages of preclinical research but could not be translated into clinical therapies. This large translational gap clearly indicates that we should be striving to develop more reliable and translational animal models of HIE and also attempt to revise those aspects of currently prevailing preparations which have little or no clinical equivalent.

It is probably no overstatement that therapeutic hypothermia has been the most important recent development in the care of infants with HIE. The concept itself, however, has been around for centuries, as the first documented attempt to use hypothermia in the resuscitation of asphyxiated infants dates back to the late 17th century. The first clinical trials of therapeutic hypothermia published encouraging results from various centers already in the 1960s. The fact that it took another half century to translate those results into clinical practice has been largely due to a combination of clinical dogmatism and historical bad luck.

However, in the last 10 years a number of randomized multicenter studies as well as large meta-analyses have established hypothermia as the standard of care for term infants with moderate-to-severe HIE. Still, the optimal timing and depth of therapeutic hypothermia remain unknown.

While hypothermia is often regarded as an artificial intervention among clinicians, there is in fact a large body of evidence from evolutionary biology showing that a hypometabolic and hypothermic response to hypoxia can be observed in the newborns of various species. In this sense hypothermia can be regarded as an evolutionarily conserved protective mechanism, which was also shown to be present in human neonates in a number of studies. Clinically, this means that therapeutic hypothermia is more of an attempt to enhance preexisting natural protective mechanisms and it was in fact artificial and counter-productive to attempt to rewarm these infants to 37 ˚C right after resuscitation, which we otherwise regard as physiological temperature. At the same time, however, preclinical research would have to follow closely and attempt to control for the effect of this hypoxic hypometabolism response in order to avoid including it as a potential confounding factor. Unfortunately, very few such studies exist which would adequately address this issue.

OBJECTIVES

The objectives of this thesis can be summarized as follows:

1. **To investigate the effects of cooling to different target temperatures in a preclinical piglet model of HIE. In particular, to examine the safety and efficacy of deep hypothermia (30 ˚C) utilizing clinical outcome markers and neuro-pathological indicators.**

2. **To examine the confounding effect of inadvertent hypothermia during and after hypoxia in a rodent model of HIE.**

3. **To characterize the novel rodent model of HIE adopted from Prof. Kai Kaila's group.**

METHODS

Piglet experiments:

All experiments were conducted under UK Home Office Guidelines. Twenty-eight large white male piglets, less than 24 h old were anesthetized, tracheostomized and intubated. Inflatable vascular occluders were applied around both common carotid arteries and umbilical venous and arterial catheters were inserted. All experimentation was conducted under isoflurane anesthesia and fentanyl analgesia. Heart rate (HR), mean arterial blood pressure (MABP) and rectal temperature (Trec) were continuously monitored and arterial blood gases were taken every 6 hours. Intravenous volume replacement and inotrope therapy was used to maintain MABP above 40 mmHg.

Transient hypoxia-ischemia (HI) was induced by inflating the vascular occluders and reducing fractional inspired oxygen (FiO_2) to 12%. Insult severity was standardized using magnetic resonance spectroscopy. Following HI and resuscitation piglets were randomized into four groups: (I) normothermia (Trec 38.5 °C throughout), or whole-body cooling between 2–26 h after the insult to (II) Trec 35 °C, (III) Trec 33.5 °C, or (IV) Trec 30 °C. At 26 h after HI, cooled piglets were rewarmed to normothermia with a maximum speed of 0.5 °C/h. Forty-eight hours following HI, piglets were euthanized with pentobarbital, the brain was fixed with cold 4% paraformaldehyde (PFA) via cardiac perfusion, removed along with major organs and processed for histology and immunohistochemistry. The measurement of serum cortisol and troponin levels as well as the histopathology and *in situ* hybridization were carried out by my colleagues and our collaborators. Data analysis was performed by an expert statistician.

Rodent experiments:

All experiments were carried out under the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary. Wistar rat pairs (Charles River Laboratories, Hungary) were time-mated and the fathers were removed from the cages on E19 of the pregnancy. On the day of birth litters were culled to 8-12 pups. On P7 pups were separated from their dams and transferred to temperature-controlled chambers with individual cells maintained at 37 +/- 0.5 ºC (except for pilot experiments). The rectal temperature of one sentinel animal from each asphyxia group was continuously monitored and used as a surrogate marker for the rectal temperature of the group.

Following a 20-minute accommodation period pups were subjected to control or asphyxic gas mixtures for 15 mins (room air or 4% O_2, 20% CO_2, 76% N_2, respectively) and were allowed to recover for 60 mins before returning to their dams. This model procedure was originally developed by Prof Kai Kaila's group at the University of Helsinki. Early outcome groups were sacrificed 24 hours after the insult, while others were allowed to reach adulthood. Following ketamine-xylazine euthanasia, brain and other major organs were collected and fixed in paraformaldehyde and blood samples were taken. Histopathological examinations were performed using Nissl staining as well as immunohistochemistry against MBP, SMI-32 and IBA-1. The surviving group of animals were tested in a battery of behavioral tests between P40 and P100. This battery included the Open Field, the Elevated Plus Maze and the Rotarod tests for investigating neuromotor function and anxiety, while the Operant Learning – Delayed Discounting paradigm was employed to study cognitive function and impulsive behavior. Statistical analysis was performed using the GraphPad Prism V software.

RESULTS

Cooling to different target temperatures in a piglet model of HIE

There were no differences in baseline characteristics between the groups. Six episodes of cardiac arrest has occurred in the 30 °C group and five of these animals has died before the end of the experiment. One animal in the 33.5 °C group also suffered two cardiac arrests, but could be resuscitated and survived until the end of the experiment. No cardiac arrest or mortality was observed in the other groups.

HR was significantly lower in the 30 °C group during cooling compared to the normothermic group, but no other differences were noted. MABP was also similar between the groups when averaged over the different time periods. However, the 30 °C group suffered periods of profound hypertension which were treated aggressively with volume replacement and inotropes but still often led to cardiac arrest. The median volume replacement and inotrope therapy were both significantly higher in the 30 °C group compared to the normothermic group and it was also only the 30 °C group, which required second- and third-line inotropic agents.

pH was significantly lower in the 30 °C group at 36 h after HI compared to the normothermic group. Base deficit was significantly increased in the 30 °C group at 12, 24 and 36 h compared to all other groups. Blood glucose was also significantly higher in the 30 °C group at 12 h compared to the normothermic and the 35 °C group. The highest blood lactate values were also recorded in the 30 °C group at 12 and 24 h, but this was not statistically significant.

Potassium levels were significantly lower in the 30 °C group at 12 h compared to the 35 °C and the 33.5 °C groups. Hematocrit and hemoglobin values were also found to be higher in the 30 °C group during the cooling period compared to the other hypothermia groups. Cortisol measurements did not reveal any significant differences between the groups, while cardiac troponin-I levels were significantly lower in the 30 °C group compared to all other groups. Macroscopic organ pathology did not show any significant differences between the groups.

The *in situ* hybridization studies were performed by our collaborators and published separately; the details of this study can be found in that article.. These investigations focused on eight mRNA transcripts relevant for perinatal brain injury (BDNF, MANF, GFAP, MAP2, HSP70, NgR, LDH-A, LDH-B) and found that most of them were significantly affected by

the HI insult. Hypothermia counteracted this affect to a certain degree in most brain areas. In some instances, it was indicated that cooling to 33.5 °C conferred the most benefit when compared to 35 °C and 30 °C. Deep hypothermia to 30 °C made the effect of HI even more severe in certain brain regions.

The histological and immunohistochemical analysis of the collected brain samples were performed by my colleagues and published separately; the details of this study can be found in that article. Briefly, markers of necrotic and apoptotic neuronal death as well as microglial activation markers showed that cooling to 35 °C and 33.5 °C both effectively ameliorated hypoxic-ischemic brain damage, while this effect was almost completely lost with 30 °C hypothermia.

To summarize, deep hypothermia to 30 °C increased mortality and morbidity following HI. These animals suffered more cardiac arrests, profound hypertension and required a significantly higher level of volume replacement and inotrope therapy. They also suffered from severe metabolic derangements. While deep hypothermia led to such severe systemic complications, no additional neuroprotective benefits could be identified and in certain brain areas even detrimental effects were observed.

Investigating the effect of endogenous hypothermia in a rodent model of HIE

During our pilot experiments, we first examined the effect of ambient temperature on the asphyxia tolerance of neonatal rat pups. We used three chamber temperature ranges: 30-31 °C, 33-34 °C, and 36-37 °C. We found that on average, insult duration could be extended to 45 minutes at 30-31 °C, 27.5 minutes at 33-34 °C and 15 minutes at 36-37 °C ambient temperatures. Considering the fixed temperature environment of intrauterine asphyxia, where the fetal temperature cannot decrease below the maternal, we chose to conduct all subsequent experiments at 37 ± 0.5 °C chamber temperatures.

A similar pattern of temperature changes was observed in all pilot temperature groups. During the pre-insult accommodation period rat pups achieved a rectal temperature which could be considered normothermic in the given ambient temperature environment. During asphyxia, however, we observed a controlled and reproducible gradual temperature decrease to the lowest possible temperature allowed by the ambient temperature. This pattern was consistent with hypoxic hypometabolism and endogenous hypothermia in this paradigm.

Characterization a novel rodent model of HIE

Following the pilot experiments, we proceeded with the characterization of this novel rodent model of HIE, which was originally developed by Prof Kai Kaila's group at the University of Helsinki. The baseline characteristics of the animals were similar between the two groups. The asphyxia group had an overall mortality of 20.7 %, while the control group suffered no mortality.

In order to assess early brain injury, animals were sacrificed and their major organs removed 24 h after asphyxia. Nissl staining did not reveal any major neuronal death throughout various brain regions. Similarly, no major white matter or axonal injury could be seen using MBP and SMI-32 staining. However, IBA-1 immunohistochemistry revealed a significantly elevated number of activated microglial cells in the prefrontal cortex and the hippocampus.

Behavioral testing in adulthood did not reveal significant differences in locomotion or motor function in either the Open Field, the Rotarod or the Elevated Plus Maze test. However, the asphyxic animals displayed increased anxiety-like behavior in the Elevated Plus Maze test. The Delayed Discounting paradigm showed similar reward learning between the two groups, whereas the asphyxia group showed a significantly higher level of motor impulsivity.

In summary, we have observed a pattern of neuroinflammation in the prefrontal cortex and the hippocampus without overt neuronal or white matter injury. This was associated with a lack of gross neuromotor dysfunction but significantly increased anxiety- and motor impulsivity-like behaviors. This phenotype is similar to human attention-deficit/hyperactivity disorder (ADHD). It has been previously suggested by epidemiological cohort studies that perinatal hypoxic events might play a role in the development of ADHD. This novel rodent model of HIE may prove useful in studying these relationships as well as potential neuroprotective interventions.

CONCLUSION

Based on the work described in this thesis, the following conclusions can be drawn:

1. **Deep hypothermia to 30 ˚C poses a significant risk for systemic side-effects and offers no additional benefit in neuroprotection in neonatal hypoxic-ischemic encephalopathy. Our results confirm that physicians now adopting therapeutic hypothermia should follow established treatment guidelines strictly, and carefully avoid inadvertent overcooling.**

2. **Hypoxic hypometabolism and endogenous hypothermia are important confounders in a preclinical rodent model of birth asphyxia. In order to maximize translational value, researchers should take specific measures to avoid unintended hypothermia during hypoxia.**

3. **This novel, non-invasive rodent model of mild perinatal asphyxia presents a neurodevelopmental and behavioral phenotype consistent with human ADHD-like symptoms. This paradigm could be valuable for developing a causal understanding between perinatal environmental effects and psycho-developmental impairments in later childhood.**

PUBLICATION LIST

A. Kerenyi, D. Kelen, SD. Faulkner, A. Bainbridge, M. Chandrasekaran, EB. Cady, X. Golay, NJ. Robertson: *Systemic effects of whole-body cooling to 35 °C, 33.5 °C, and 30 °C in a piglet model of perinatal asphyxia: implications for therapeutic hypothermia*, Pediatric Research, 2012

L. Olson, S. Faulkner, K. Lundströmer, **A. Kerenyi**, D. Kelen, M. Chandrasekaran, U. Ådén, L. Olson, X. Golay, H. Lagercrantz, NJ. Robertson, D. Galter: *Comparison of Three Hypothermic Target Temperatures for the Treatment of Hypoxic Ischemia: mRNA Level Responses of Eight Genes in the Piglet Brain*, Translational Stroke Research, 2013

D. Alonso-Alconada, KD. Broad, A. Bainbridge, M. Chandrasekaran, SD. Faulkner, **A. Kerenyi**, J. Hassell, E. Rocha-Ferreira, M. Hristova, B. Fleiss, K. Bennett, D. Kelen, E. Cady, P. Gressens, X. Golay, NJ. Robertson: *Brain cell death is reduced with cooling by 3.5°C to 5°C but increased with cooling by 8.5°C in a piglet asphyxia model*, Stroke, 2014

The results of the rodent experiments described in this thesis are as of yet unpublished.