Differential effects of hypothermia on early and late epileptiform events after severe hypoxia in preterm fetal sheep

Bennet L, Dean JM, Wassink G, Gunn AJ

Depts of Physiology and Paediatrics, University of Auckland, Auckland, New Zealand.

Running Title: Hypothermia and EEG transients

Mailing Address:

Alistair Jan Gunn,
Dept of Physiology,
Faculty of Medicine and Health Science
The University of Auckland
Private Bag 92019
Auckland, New Zealand
Phone: 64 9 373-7599 ext 86763
Fax: 64 9 373-7499
Email: aj.gunn@auckland.ac.nz
Abstract

Moderate cerebral hypothermia is consistently neuroprotective after experimental hypoxia-ischemia; however, its mechanisms remain poorly defined. Using a model of complete umbilical cord occlusion for 25 min in 0.7 gestation fetal sheep, we examined the effects of cerebral hypothermia (fetal extradural temperature reduced from 39.5±0.2°C to less than 34°C), from 90 min to 70 h after the end of the insult, on post-occlusion epileptiform activity. In the first 6 h after the end of occlusion fetal EEG activity was abnormal with a mixture of fast and slow epileptiform transients superimposed on a suppressed background; seizures started a mean of 8 h after occlusion. There was a close correlation between numbers of these EEG transients and subsequent neuronal loss in the striatum after 3 days recovery ($r^2=0.65$, $P=0.008$). Hypothermia was associated with a marked reduction in numbers of epileptiform transients in the first 6 h, reduced amplitude of seizures and reduced striatal neuronal loss. In conclusion, neuroprotection with delayed, prolonged head cooling after a severe asphyxial insult in the preterm fetus was associated with potent, specific suppression of epileptiform transients in the early recovery phase, but not of numbers of delayed seizures.

Keywords: Asphyxia – prematurity – hypothermia, induced – EEG – epileptiform transients
Introduction

Exposure to perinatal hypoxic-ischemic injury continues to be a significant cause of long-term neurodevelopmental impairment. Although the etiology of brain injury in premature infants is highly complex, and multifactorial, ‘larger’ premature infants, from 31 to 36 gestation, with metabolic acidosis on umbilical cord blood have a high rate of evolving encephalopathy (Salhab and Perlman 2005) and subsequent basal ganglia damage (Barkovich and Sargent 1995), and thus, may be candidates for experimental neuroprotective treatments (Salhab and Perlman 2005). Consistent with clinical and experimental evidence for neuroprotection with cerebral hypothermia at term (Gluckman et al. 2005; Gunn and Thoresen 2006; Shankaran et al. 2005), we have recently reported that delayed, prolonged moderate head cooling can improve survival of phenotypic striatal projection neurons after severe hypoxia in the preterm sheep fetus (George et al. 2006). The mechanisms of hypothermic protection in general are only partially understood, particularly in the very immature brain.

Although the precise mechanisms of preterm neural injury are extremely complex, many studies have suggested that excitotoxic activity is a key factor, as recently reviewed (du Plessis and Volpe 2002). Studies in adult models suggest that toxicity after reversible global insults is mediated by a post-ischemic increase in glutamate receptor-mediated excitatory responses rather than by high extracellular concentrations of glutamate (Kalemenev et al. 2002; Mitani et al. 1998). The immature brain has been found to be particularly susceptible to such post-hypoxic receptor hyperexcitability (Jensen and Wang 1996). Consistent with this, we have reported in a paradigm of severe hypoxic-ischemic brain injury in the immature fetal sheep, that although overall EEG activity was profoundly suppressed for many hours after the asphyxial insult regardless of whether injury later developed or not, epileptiform EEG
Transient activity was only seen in the early recovery phase after a severe insult that was associated with severe injury (George et al. 2004). The maximal frequency of these events corresponds with a fall in cerebral oxygenation on near-infrared spectroscopy (Bennet et al. 2006). These EEG transients were low to moderate amplitude events similar to the subtle, brief spikes and waveforms observed in preterm infants (Scher et al. 1994). Clinically, the frequency of such epileptiform EEG transients is strongly associated overall with poor outcome (Biagioni et al. 2000; Hughes and Guerra 1994; Marret et al. 1997; Okumura et al. 2003; Rowe et al. 1985; Vecchieri-Blineau et al. 1996).

Clearly these EEG transients could simply be a manifestation of injury rather than a cause. A possible causal relationship is supported, however, by the observations that suppression of EEG transients with a glutamate receptor antagonist reduces cell loss (Dean et al. 2006a), and conversely that increased EEG transient activity during blockade of inhibitory α2-adrenergic receptor activity was associated with increased neuronal loss (Dean et al. 2006b). Further, the maximal frequency of EEG transients in the preterm sheep model preceded secondary failure of mitochondrial oxidative activity on continuous near-infrared spectroscopic monitoring, whereas overt seizures did not occur until after the onset of the secondary fall (Bennet et al. 2006). There is considerable evidence suggesting that delayed failure of mitochondrial function is closely linked with the development of cell death (Vannucci et al. 2004).

While the effect of hypothermia on post hypoxic-ischemic EEG transients per se is unknown, hypothermia reduces the slope of excitatory postsynaptic potentials (Aihara et al. 2001), and focal cerebral cooling can reduce epileptiform activity in animals (Baldwin and Frost 1956) and in patients (Karkar et al. 2002). Further, hypothermia slows the rate of propagation and frequency of induced spreading depression within the cortex of adults rats (Takaoka et al. 1996; Ueda et al. 1997). Thus, these data suggest the hypothesis that suppression of EEG
transients by hypothermia may be a significant mechanism of hypothermic protection from evolving hypoxic-ischemic injury in the immature brain.

The aim of this study was to determine whether following hypoxic-ischemic injury induced by profound asphyxia in the 0.7 gestation fetal sheep, treatment with cerebral hypothermia, induced 90 min after reperfusion and continued for 3 days, would be associated with suppression of post-hypoxic-ischemic EEG transients on continuous EEG monitoring. In terms of cerebral maturity the 0.7 gestation fetal sheep is comparable to the human brain at 28-32 weeks of gestation, prior to the onset of cortical myelination (Barlow 1969).
Materials and Methods

We have previously reported changes in temperature, blood gases and pH, insulin-like growth factors and their binding proteins, and survival of phenotypic neurons in an overlapping subset of the animals used in the present analysis (George et al. 2006; Roelfsema et al. 2005). Blood gas samples were analyzed at the core temperature of the fetal sheep at the time of collection.

Experimental preparation

All procedures were approved by the Animal Ethics Committee of The University of Auckland. Singleton Romney/Suffolk fetal sheep were instrumented at 97-99 days of gestation (term = 147 days) under general anesthesia (2% halothane in O₂) using sterile techniques. Catheters were placed in the left femoral artery and vein, right brachial artery and vein, and the amniotic sac. Two pairs of electroencephalogram (EEG) electrodes (AS633-5SSF, Cooner wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 15 mm anterior to bregma and 10 mm lateral), with a reference electrode sewn over the occiput. A thermistor (Incutemp-1, Mallinckrodt Medical Inc., St Louis, MO) was placed over the parasagittal dura 20 mm anterior to bregma, to measure extradural temperature, and the burr holes were sealed and the skin over the fetal skull was secured with cyanoacrylate glue. Electrocardiogram (ECG) electrodes were sewn across the chest to record the fetal heart rate. A second thermistor (to measure fetal core body temperature) was placed in the fetal esophagus at the level of the right atrium. An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA).

A cooling coil made from silicone tubing (external diameter 7.9 mm, internal diameter 4.8
mm; Silclear, Degania Silicone, Degania Bet, Israel) was attached over the dorsal surface of the scalp and extended over the lateral surface of the cranium down to the level of the external auditory meatus. The fetus was returned to the uterus and all fetal leads were exteriorized through the maternal flank. The maternal long saphenous vein was catheterized to provide access for maternal care and euthanasia. Antibiotics were given to the ewes, prior to the start of surgery (5 ml of Streptopen, i.m.), into the amniotic sac prior to closure of the uterus (80 mg Gentamicin), and after surgery (80 mg Gentamicin i.v. daily for the first 2 days, and 600 mg Crystapen i.v. for 4 days).

After surgery, sheep were housed together in separate metabolic cages with access to water and food ad libitum. They were kept in a temperature-controlled room (16±1°C, humidity 50±10%), in a 12 h light/dark cycle. A period of 4-6 days post-operative recovery was allowed. Catheters were maintained patent by continuous infusion of heparinized saline (20 U ml⁻¹ at 0.2 ml h⁻¹).

*Experimental design and recordings*

Experiments were conducted at 103-104 days gestation. Fetal mean arterial pressure (MAP), corrected for maternal movement by subtraction of amniotic fluid pressure, heart rate (FHR) and EEG activity were recorded continuously from 12 h before the experiment until 72 h afterwards. Data were collected by computer and stored to disk for off-line analysis (Labview for Windows, National Instruments Ltd., Austin, Texas, USA).

Fetuses were randomly assigned to either normothermia-occlusion (n=8), hypothermia-occlusion (n=9), or sham control (n=7) groups. Fetal asphyxia was induced in both occlusion groups by rapid inflation of the umbilical cord occluder for 25 min with sterile saline of a defined volume known to completely inflate the occluder. Successful occlusion was
confirmed by observation of a rapid flattening of EEG amplitude. In all groups fetal arterial blood was taken at 15 min prior to asphyxia, 20 min during asphyxia, and 2, 4, 6, 24, and 72 h post-asphyxia for blood gas, acid-base balance (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, MA, USA) and for glucose and lactate determination (YSI model 2300, Yellow Springs, Ohio, USA). Cerebral cooling was performed from 90 min to 70 h after the end of occlusion. Fetuses were then allowed to spontaneously rewarmed for 2 h. Cooling was induced by circulating cold water (10°C) through a coil around the fetal head (George et al. 2006; Gunn et al. 1997). Cooling was titrated in the first 2 h to reduce fetal extradural temperature from 39.4±0.1°C to less than 34°C.

Fetuses were studied for 3 days after the end of occlusion. On completion of the experiment the ewes and fetuses were killed by an overdose of sodium pentobarbitone (9 g, i.v. to the ewe: Pentobarb 300, Chemstock International, Christchurch, New Zealand).

**Histology tissue preparation**

Fetal brains were perfusion fixed *in situ* with normal saline followed by 500 ml of 10% phosphate buffered formalin. Following removal from the skull, tissue was fixed for a further 5-6 days before processing and embedding using a standard paraffin tissue preparation (Gunn et al. 1997). Neuronal loss was evaluated by light microscopy by an assessor masked to the treatment group on 6 μm thick coronal sections stained with thionin and acid fuchsin (George et al. 2006; Gunn et al. 1997). The proportion of neurons showing ischemic cell change as shown by nuclear condensation and acid fuchsin (pink) staining of the cytoplasm in multiple pre-assigned areas was scored on a 6 point scale as follows: 0 = no dead neurons; 5 = >0–10%; 30 = >10–50%; 70 = >50–90%; 95 = 90–<100%; and 100 = 100% dead neurons. Average scores were calculated for each region.

**Data analysis and statistics**
Off-line analysis of electronic data was performed using a custom analysis program based on Labview for Windows. The analogue EEG signal (recorded using amplifier filter modules kindly supplied by Brainz Ltd, Auckland, New Zealand) was processed with a first-order high-pass filter at 1.6 Hz and a 6th order Butterworth low-pass filter with a cut-off frequency of 50 Hz and then digitally stored at a sampling rate of 64 Hz for analysis of seizures. The raw EEG was assessed for epileptiform activity; specifically the presence of spikes and sharp waves (i.e. epileptiform transients) (Bennet et al. 2006; George et al. 2004). A spike was defined as having a sharp outline and duration of less than 70 msec. Sharp waves were assessed as single or repeated mono- or diphasic transients lasting 100 to 250 msec, with an amplitude greater than 10 $\mu$V, typically superimposed on a flat EEG background (Scher 2002). Overt electrographic seizures were identified visually and defined as the concurrent appearance of sudden, repetitive, evolving stereotyped waveforms in the EEG signal lasting more than 10 sec (Scher 2002). All analysis of epileptiform activity was performed by a team member who was masked to the study design (LB).

Treatment effects were evaluated by analysis of variance (ANOVA, SPSS v12, SPSS Inc., Chicago, IL), followed by the protected least-significant difference post-hoc test when a significant overall effect was found. The incidence of seizures was compared by Mann-Whitney U test. The relationship between epileptiform events and neuronal loss was assessed by linear regression analysis. Statistical significance was accepted when $P<0.05$. Data are mean±SD.
Results

All fetuses had normal FHR, MAP, blood gases, acid base, glucose and lactate status before each experiment, according to the standards of our laboratory (Table 1). Occlusion of the umbilical cord was associated with marked fetal hypoxia and acidosis (Table 1), bradycardia and hypotension ($P<0.001$, data not shown), similar to our previous reports in this model (Bennet et al. 2006; George et al. 2004). Following reperfusion, there was rapid recovery of FHR and MAP. MAP was significantly higher one hour after the end of occlusion in both occlusion groups compared with the sham control group ($P<0.005$), and returned to sham control values by 12 h. There was no significant difference between the hypothermia-occlusion group and the normothermia-occlusion group.

Effect of cooling on brain and body temperature

Head cooling was initiated 90 min after the end of the umbilical cord occlusion and was associated with a significant fall in extradural temperature starting within an hour after the onset of cooling, reaching $30.1\pm2.5^\circ\text{C}$ after 2 h ($vs$ $39.5\pm0.2$; $P<0.001$). Extradural temperature gradually increased during the second and third day of cooling to a mean of $32.3\pm2.2^\circ\text{C}$ in the last 6 h. During cooling, core body temperature dropped mildly to $37.5\pm0.7$ ($vs$ $39.4\pm0.3$; $P<0.01$) after 2 h, with no further significant change over the cooling period (Roelfsema et al. 2005).

Histology

As previously reported (George et al. 2006), umbilical cord occlusion with normothermic recovery was associated with severe subcortical neuronal loss, particularly in the dorso-lateral aspect of the basal ganglia; there was no histological injury or cell loss in sham controls. Hypothermia was associated with a significant overall reduction in striatal neuronal loss.
(37.7±17.3% vs 5.5±3.5%, P<0.001).

**Epileptiform activity**

Following occlusion, the raw EEG signal was highly abnormal showing marked loss of background activity, with frequent high voltage sharp and spike wave transients (Fig. 1). These EEG transients peaked between 3 and 5 h after the end of occlusion. No abnormal EEG waveforms, or burst suppression activity was observed in the sham control group at any time. The start of cooling at 90 min was associated with an immediate reduction in numbers of EEG transients (P<0.001, Fig. 1). Although there was a trend for increasing numbers of transients in the hypothermia group after approximately 5 h, the onset of overt EEG seizures at this time in some fetuses meant that EEG transients could no longer be reliably counted. Within the normothermia-occlusion group there was a close correlation between maximal numbers of events in the first 6 h and neuronal loss in the striatum (r² = 0.65, P=0.008, Fig. 2); there was no significant correlation within the hypothermia-occlusion group alone.

This interval of fast and sharp wave spike activity was then followed by the onset of high amplitude, low frequency evolving seizure activity starting 8.3±5.2 h after end of occlusion in the normothermia-occlusion group vs 8.5±1.5 h in the hypothermia-occlusion group (N.S.). A total of 63±54 vs 73±70 seizure events occurred (N.S., Mann-Whitney U test). The amplitude of these events was reduced in the hypothermia-occlusion group (135±59 vs 67±21 μV, P=0.006, Mann-Whitney U test; Fig. 3). No seizures were seen in either occlusion group in the 2 h rewarming period, and no seizures were observed in sham control fetuses at any time.
Discussion

The present study demonstrates that striatal neuroprotection with moderate, delayed cerebral hypothermia from 90 minutes to 70 hours after a prolonged episode of umbilical cord occlusion was associated with marked suppression of numbers of early onset epileptiform EEG transients. In contrast, hypothermia had no effect on numbers of subsequent, delayed electrographic seizures, although the mean amplitude of the seizures was significantly reduced. Basal ganglia damage due to perinatal asphyxia represents a distinct and under-appreciated cause of preterm brain injury (Barkovich and Sargent 1995; Gilles et al. 1998; Leijser et al. 2004; Lin et al. 2001; Paneth et al. 1990), particularly in ‘larger’ premature infants (Salhab and Perlman 2005). In the long-term there is an independent association between impaired growth of grey matter nuclei and functional impairment, even after adjustment for white matter injury (Woodward et al. 2005).

The present study provides further support for the concept that abnormal neural activity after hypoxia-ischemia may contribute to delayed cell loss in the immature brain. Consistent with previous reports in this paradigm (Bennet et al. 2006; Dean et al. 2006a; Dean et al. 2006b; George et al. 2004), despite profound suppression of normal background EEG activity, frequent spikes and sharp and slow wave complexes were seen in the early recovery phase after umbilical cord occlusion, well before overt seizures developed. In the current study, we report for the first time that the frequency of these EEG epileptiform transients was highly associated with the severity of subsequent striatal damage in normothermic animals. This finding suggests that these events originate in subcortical structures and propagate to the cortex (de Haan et al. 1997). Critically, the reduced frequency of these EEG transients during moderate hypothermia was associated with markedly reduced striatal damage. Although this association further strengthens the hypothesis that these events may be pathogenic it is not yet
sufficient to establish causality; further studies are needed to examine whether the same relationship exists in different models and during other manipulations that increase or decrease numbers of transients.

The effect of hypothermia on EEG transients is consistent with the well established effect of hypothermia to reduce the slope of excitatory postsynaptic potentials \textit{in vitro} (Aihara et al. 2001) and of glutamate release after depolarization (Nakashima and Todd 1996) and following reperfusion from severe hypoxia-ischemia in the piglet (Thoresen et al. 1997). Focal cerebral cooling can reduce epileptiform activity in animals (Baldwin and Frost 1956) and in patients (Karkar et al. 2002) without changing the motor threshold for electrical stimulation. Nevertheless, it is unlikely that neuroprotection with hypothermia is solely due to suppression of N-methyl-D-Aspartate receptor activity, as improvement in neuronal loss with hypothermia in the present study is markedly greater than we observed after an infusion of the highly selective antagonist dizocilpine in this model (Dean et al. 2006a).

A further mechanism by which hypothermia may protect the brain from secondary extension of ischemic injury is inhibition of spreading depression (Ueda et al. 1997). Spreading depression is a rapid and nearly complete depolarization of a sizable population of brain cells with massive redistribution of ions between intracellular and extracellular compartments, which propagates in a wave (Somjen 2001). There is increasing evidence that spreading depression-like events during ischemia increase the extent of neural injury by altering ion gradients or increasing glutamate release (Hossmann 1996; Mies et al. 1993). In contrast, the role of these events after hypoxia-ischemia is not well defined. Intriguingly, there is \textit{in vitro} evidence that the juvenile brain is more likely than the adult to show spontaneous spreading depression-like events during recovery from hypoxia (Luhmann and Kral 1997). Further, whereas these events are not injurious to the normoxic adult brain (Somjen 2001), in juvenile
normoxic hippocampal slice cultures repetitive spreading depression-like events led to
deterioration of evoked fast field potentials and cell damage (Pomper et al. 2006).

Given that spreading depression-like events have been commonly associated with
epileptiform activity, and may further increase the excitability in human brain tissue (Gorji
and Speckmann 2004), we speculate that such events may accompany epileptiform
transient activity in this model, and exacerbate injury; further studies are needed to assess
this. Mild hypothermia slows the rate of propagation and frequency of spreading depression-
like events in adults rats both after direct induction (Takaoka et al. 1996; Ueda et al. 1997)
and during focal ischemia (Chen et al. 1993; Mancuso et al. 2000). Consistent with this,
during focal ischemia in adult rats mild hypothermia decreased the frequency of spreading
depression-like events (Chen et al. 1993) and associated transient reductions in apparent
diffusion co-efficient of water (Mancuso et al. 2000).

In our study, the effect of hypothermia seemed to be specific to early EEG epileptiform
transients, since continued cooling did not affect the numbers or time of onset of late onset
seizures that were seen from a mean of 9 hours after occlusion. This is consistent with
previous studies which have reported that hypothermia is associated with either no significant
effect on secondary seizures (Gunn et al. 1997; Gunn et al. 1998), or a moderate reduction in
numbers of electrical seizures (Tooley et al. 2003). This is the first perinatal study to report
that hypothermia had a selective effect to reduce the amplitude of post-hypoxic seizures.
Although the onset of seizures was not until after the known onset of the secondary
deterioration in mitochondrial function in this paradigm (Bennet et al. 2006), we cannot
exclude the possibility that the reduction in seizure intensity may have contributed to
improved recovery. Very prolonged, intense induced seizures can unquestionably cause
neural injury (Wasterlain et al. 2002). Further, delayed post-ischemic seizures were
associated with a marked extracellular accumulation of potentially damaging excitatory amino acids in near-term fetal sheep (Tan et al. 1996a), and conversely hypothermia after hypoxia-ischemia in the piglet reduced the secondary rise in excitotoxins (Thoresen et al. 1997).

Nonetheless, the immature brain appears to be much more resistant to seizure induced damage per se than in adulthood (Fernandes et al. 1999; Haas et al. 2001; Lado et al. 2000). Consistent with a limited pathogenic role for delayed seizures, treatment with hypothermia after severe cerebral ischemia in term-equivalent fetal sheep was highly protective if it was started in the early recovery phase (Gunn et al. 1997), but not if initiation of cooling was delayed until shortly after the start of post-ischemic seizures (Gunn et al. 1999). Similarly, complete suppression of post-ischemic seizures with an infusion of a glutamate antagonist started just before the average onset time of seizures, in the same model, did not reduce parasagittal cortical infarction, and was associated with only a modest reduction in neuronal loss in other regions (Tan et al. 1996b). It is intriguing to note that consistent with our findings, preventing spontaneous hyperthermia reduced the amplitude of kainic acid induced seizures after hypoxia-ischemia in 10 day old rat pups and abolished the increase in neural injury (Yager et al. 2004). These data reinforce the concept that even intense excitotoxin related seizures may not directly augment post-hypoxic injury.

In summary, this study demonstrates that the severity of neuronal loss in developing basal ganglia after profound asphyxia is highly correlated with the frequency of epileptiform transients in the early recovery period. Neuroprotection with cerebral hypothermia after profound asphyxia was associated with marked suppression of these events, whereas there was only a reduction in the amplitude, but not the time of onset or numbers of later onset seizures. These data support the hypothesis that EEG transients are associated with neural
injury and may be analogous to the phenomena of spreading depression. It is now important to investigate how this association is affected by a wider range of manipulations and whether monitoring for such abnormal activity may be of assistance in identifying potentially treatable phases after exposure to hypoxic-ischemia in the developing brain.

Acknowledgements

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Figure Legends

Figure 1. Examples of raw electroencephalogram (EEG) data showing normal discontinuous mixed frequency EEG activity (panel A), epileptiform transients superimposed on a suppressed EEG background at 3 h post-occlusion (panel B) and marked suppression of EEG transients during hypothermia at 3 h post-occlusion (panel C). The bottom panel (panel D) quantifies numbers of epileptiform transients (spikes, and sharp and slow waves) in 30 minute intervals in the normothermia-occlusion and hypothermia-occlusion groups. * \( P<0.05 \) hypothermia-occlusion vs normothermia-occlusion. Data are mean±SD.

Figure 2. The relationship between maximal frequency of EEG epileptiform transient events in 30 minute intervals in the first 6 h after umbilical cord occlusion, and neuronal loss in the striatum after 3 days recovery.

Figure 3. Examples of raw fetal electroencephalogram (EEG) demonstrating evolving seizure activity following occlusion in normothermia-occlusion (panel A) and normothermia-hypothermia groups (panel B). Note the reduced amplitude of the seizure during head cooling (panel B).
Table Legends

Table 1. Fetal arterial pH, blood gases, glucose and lactate values for sham control (C), normothermia-occlusion (N) and hypothermia-occlusion (H) groups 15 min before (baseline), at 20 min during (occl), and 2 h, 4 h, 6 h, 24 h, and 72 h after either sham occlusion or 25 min of umbilical cord occlusion. Data are mean±SD. * P<0.05 compared to sham control, # P<0.05 normothermia-occlusion vs hypothermia-occlusion (ANOVA, followed by the Least Significant Difference post-hoc test for between group comparisons).
Table 1.

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<td>N</td>
<td>H</td>
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<td>1.2±0.5*,#</td>
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