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Minimal systemic hypothermia combined with selective head cooling evaluated in a pig model of hypoxia-ischemia

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BACKGROUND: Selective head cooling (SHC) with moderate hypothermia (HT) and whole-body cooling are beneficial following perinatal asphyxia. SHC with systemic normothermia (NT) or minimal HT is under-investigated, could obviate systemic complications of moderate HT, and be applicable to preterm infants. We hypothesized that minimal systemic HT with SHC following hypoxia-ischemia (HI) would be neuroprotective compared with systemic NT.

METHODS: Newborn pigs underwent global HI causing permanent brain injury before being randomized to NT (rectal temperature (T_{rectal}) 38.5 °C) or minimal HT (T_{rectal} 37.0 °C) with SHC (cooling cap and body wrap) for 48 h followed by 24-h NT with 72-h survival.

RESULTS: SHC did not reduce global or regional neuropathology score when correcting for insult severity or compared with a NT group matched for HI severity but increased mortality by 26%. During 48 h, the SHC mean ± SD T_{rectal} was 37.0 ± 0.2 °C, and $T_{deep\ brain}$ and $T_{superficial\ brain}$ were 35.0 ± 1.1 °C and 31.5 ± 1.6 °C, respectively, with stable T_{brain} achieved ≥3 h after starting cooling.

CONCLUSION: This is the first study in newborn pigs of minimal systemic HT with SHC for 48 h and a further 24 h of NT following HI. Mortality was increased in the cooled group with no neuroprotection in survivors.

Therapeutic hypothermia (HT) using whole-body cooling (WBC) to a rectal temperature (T_{rectal}) of 33.5 °C or selective head cooling (SHC) with moderate systemic HT to 34.5 °C is the only effective treatment for hypoxic ischemic encephalopathy (HIE) in term infants (1). HIE also occurs in preterm infants and HT trials are planned (e.g., Preemie Hypothermia for Neonatal Encephalopathy, NCT01793129) but concerns remain about systemic adverse effects (2–4). SHC without moderate systemic HT is under-investigated and is suggested to reduce potential systemic complications of whole body-HT. Reducing deep-brain temperature ($T_{deep\ brain}$) using SHC in term infants is difficult but may be easier in preterm infants with their smaller head size, thinner skin, and skull (5). In newborn pigs, $T_{deep\ brain}$ is within 1 °C of core temperature at normothermia (NT) but during SHC there is a gradient of between 3 and 7 °C (6–9). SHC may offer an advantage over WBC because different brain regions may have different optimum temperatures for neuroprotection (9–11).

Short-term SHC with systemic NT is possible experimentally but neuroprotection has not been assessed using an adequate treatment period using clinically available equipments (12–14). We showed previously that SHC combined with moderate systemic HT to 33.5 °C was neuroprotective using a global model of perinatal hypoxia-ischemia (HI) in the newborn pig (8). We hypothesized that minimal systemic HT (1.5 °C below core temperature) combined with SHC for 48 h would be neuroprotective compared with systemic NT using a validated neuropathology score.

RESULTS

There were no differences in age, weight, sex distribution or baseline arterial pH, lactate, and blood glucose between groups (**Table 1**).

Mortality and Neuropathology

The HT group was compared with two NT groups: NT_R, which was randomized to the HT group, and NT_M, which was severity-matched to the survivors in the HT group. Mortality was higher in the HT compared with the NT_R group (43 vs. 17%, $P < 0.01$), usually due to cardiovascular collapse and the mean ± SD time until death was 53 h ± 7 (NT_R) and 40 h ± 24 (HT). Animals with severe insults (total duration of low-amplitude electroencephalogram (LAEEG) >30 min out of 45-min HI) did not tolerate 48 h of HT treatment and died. Where available, all animals had histological evidence of brain injury (**Figure 1**). There was neither skin injury nor injury or bleeds from the subcutaneously placed needle electroencephalogram (EEG) electrodes. The average neuropathology score in the HT group was lower than that of the NT_R group, but neuropathology was incomplete in a number of HT animals because of early death (before the end of the 48-h treatment period). The total duration of LAEEG, which is the best predictor of brain injury in this model (15), was shorter for survivors in the HT group compared with those of the NT_R group. We used a stepwise linear regression analysis to account for the discrepancy in LAEEG, which revealed arterial pH at the end of HI

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Table 1. Clinical characteristics of treatment groups

Treatment group	NT_R	NT_M	HT	*P* value
n	12	14	10	—
Female (%)	58	53	40	0.7
Age, h, mean ± SD	16 ± 5	18 ± 4	15 ± 6	0.7
Weight at start of experiment, kg, mean ± SD	1.557 ± 0.25	1.654 ± 0.27	1.655 ± 0.24	0.6
pH at baseline, mean ± SD	7.47 ± 0.07	7.48 ± 0.10	7.52 ± 0.05	0.3
Lactate at baseline, mmol/l, mean ± SD	2.6 ± 1.2	2.7 ± 1.3	3.2 ± 1.2	0.5
Blood glucose at baseline, mmol/l, mean ± SD	6.3 ± 1.6	7.6 ± 1.4	7.1 ± 1.4	0.2
Duration of LAEEG during insult, min, mean ± SD	36.7 ± 8.2	23.8 ± 2.9	21.8 ± 3.5	<0.0001*
pH at end of insult, mean ± SD	7.04 ± 0.16	7.10 ± 0.14	7.08 ± 0.12	0.5
Lactate at end of insult, mmol/l, mean ± SD	15.0 ± 1.9	19.1 ± 3.5	17.2 ± 2.1	0.006[a]
MABP during insult, mmHg, mean ± SD	37 ± 7	45 ± 7	40 ± 4	0.007[b]

n, number of animals with neuropathology available; HT, hypothermia; LAEEG, low-amplitude electroencephalogram; MABP, mean arterial blood pressure; NT_M, matched normothermia; NT_R, randomized normothermia; SD, standard deviation.
[a]Lactate at end of insult, NT_R vs. NT_M, $P = 0.002$; [b]MABP during insult, NT_R vs. NT_M, $P = 0.002$. *Duration of LAEEG during insult: NT_R vs. NT_M, $P \le 0.0001$; NT_R vs. HT, $P \le 0.0001$.

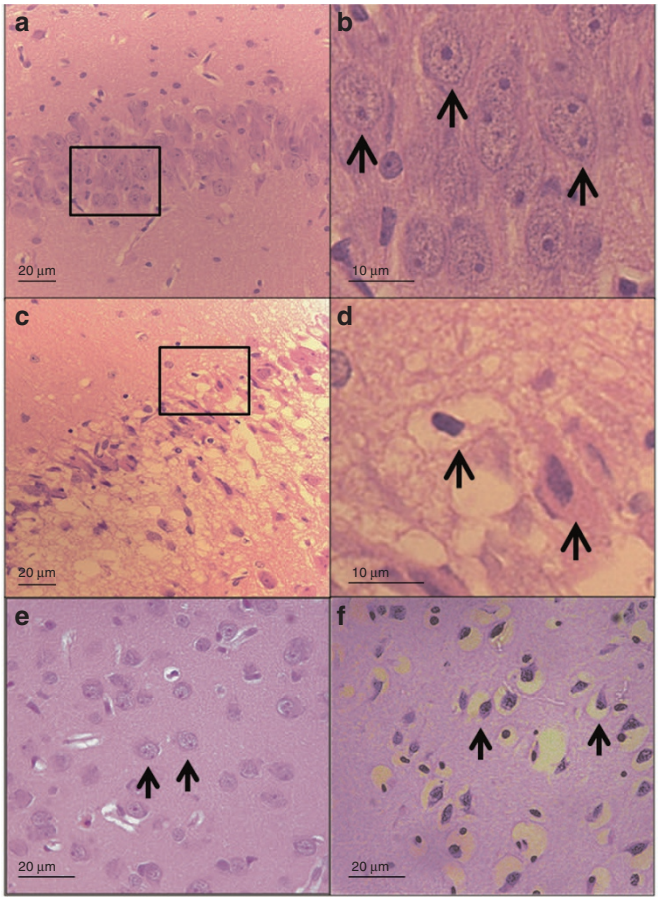

Figure 1. Representative photomicrographs of the hippocampus CA1 and putamen stained with hematoxylin and eosin. (**a**) Hippocampus CA1 region (×400 magnification) showing normal appearance and architecture with (**b**) detail showing healthy neurons (arrows). (**c**) Severe ischemic changes in hippocampus with (**b**) detail showing cytoplasmic shrinkage, nuclear condensation, disruption of tissue architecture, perineuronal vacuolation, and neuropil vacuolation (arrows). (**e**) Putamen (×630 magnification) with healthy neuronal cells (arrows) and (**f**) ischemic cells in putamen (arrows).

and total duration of LAEEG during HI as significant independent variables predicting the global neuropathology score (Table 2), but there was no significant relationship between the temperature (NT vs. HT) and the global neuropathology score. We corroborated our results by comparing the HT group with a group of historical controls (NT_M) matched for the total duration of LAEEG during HI and arterial pH at the end of HI as detailed in the Methods section. There was no difference in the total duration of LAEEG during HI, arterial pH and lactate at the end of HI, and mean arterial blood pressure (MABP) during HI between the matched groups. There were no significant differences in global or regional neuropathology scores between the NT_M and HT groups (Figure 2).

Seizures

Seizures were treated as per the drug protocol and there were no differences in clinical or electrographic seizure incidence between groups (NT_R: 6 out of 12, NT_M: 5 out of 14, and HT: 1 out of 10). The presence of seizures and seizure duration were not found to be significant independent predictors of neuropathology score in the stepwise linear regression analysis.

Table 2. Stepwise linear regression model for global neuropathology score in randomized NT_R ($n = 12$) and HT ($n = 10$) groups

Variable	*B*	SE *B*	*β*	*t*	*P*
(Constant)	23.25	8.17	—	2.85	0.010
Arterial pH at end of insult	−3.33	1.15	−0.438	−2.90	0.009
Total duration LAEEG during insult	0.05	0.017	0.406	2.70	0.014
Model $R^2 = 0.56$, adjusted $R^2 = 0.49$					

Dependent variable: global neuropathology score; excluded independent variables: temperature (NT or HT), weight, lactate, and base deficit at the end of HI, MABP, and T_{rectal} during HI insult.
B, unstandardized coefficient; *β*, standardized coefficient; SE *B*, standard error; *t*, *t*-test statistic.

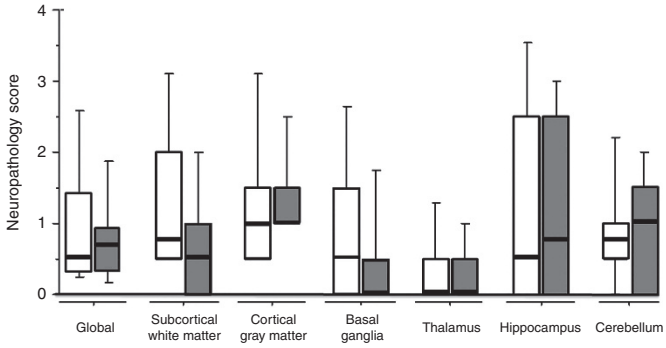

Figure 2. Global and regional neuropathology scores. Box and whisker plot showing median (thick line) and, 25th and 75th (box), and 10th and 90th (whisker) centiles for neuropathology score following 45-min HI for the NT_M (white, $n = 14$) and HT groups (gray, $n = 10$).

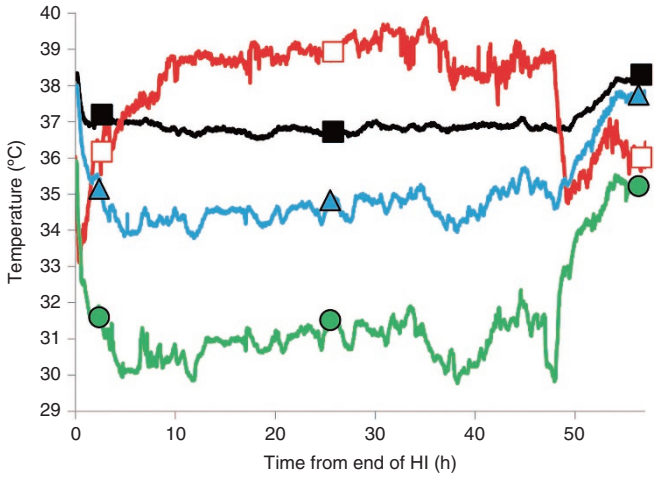

Figure 3. Mean T_{rectal}, T_{skin}, and T_{brain} during HT ($n = 14$). Mean temperatures during HT from the end of HI until the end of rewarming for the group. T_{rectal} (black, closed square), T_{skin} (red, open square), $T_{deep\ brain}$ (blue, triangle), and $T_{superficial\ brain}$ (green, circle).

Temperature

The mean ± SD T_{rectal} during cooling was 37.0 ± 0.2 °C and remained relatively stable with mean $T_{deep\ brain}$ of 35.0 ± 1.1 °C and $T_{superficial\ brain}$ of 31.5 ± 1.6 °C (Figure 3). A gradient developed between T_{rectal} being warmest, $T_{deep\ brain}$ being cooler, and $T_{superficial\ brain}$ being coolest with stable brain temperatures achieved 3–4 h after initiating cooling. The cooling cap water temperature started at 30 °C and was gradually decreased to 18 °C whilst maintaining T_{rectal} at 37 °C. There was a strong negative correlation between the T_{rectal} to $T_{superficial\ brain}$ gradient and the subcortical white matter neuropathology score in HT animals ($r = 0.7$), i.e., the colder the superficial brain, the lesser the subcortical white matter injury (Figure 4). Rewarming after 48-h HT was associated with a rapid increase in brain temperature (Figure 5). $T_{deep\ brain}$ and $T_{superficial\ brain}$ increased by around 1.8 °C/h and 4.6 °C/h, respectively. Opening the body wrap, which applied body heating during SHC, decreased T_{rectal}, $T_{deep\ brain}$, and $T_{superficial\ brain}$ by 2, 2.5, and 3.5 °C, respectively, below steady state showing that using this technique combined with the cooling cap the balance is easily disturbed by opening the body wrap. The increase in T_{rectal} back to baseline (37 °C) (Figure 5b) was accompanied by the rapid increase in $T_{deep\ brain}$ (3 °C/h) and $T_{superficial\ brain}$ (4.5 °C/h).

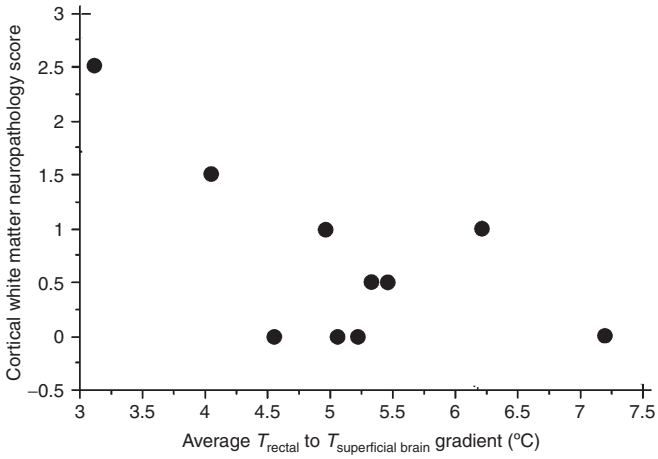

Figure 4. Relationship between average T_{rectal} to $T_{superficial\ brain}$ gradient and subcortical white matter neuropathology score. Scatter plot of individual HT animals ($n = 10$).

Cardiovascular Physiology

The mean ± SD heart rate (HR) during treatment was significantly lower in the HT group compared with that of both NT groups (NT_R: 186 ± 29, NT_M: 186 ± 21, and HT: 124 ± 21 bpm, $P < 0.05$), however, similar at the end of the experiment when all groups were at NT. The mean MABP ± SD during treatment (0–48 h after HI) was similar between the groups (NT_R: 60 ± 10, NT_M: 52 ± 6, and HT: 51 ± 6 mmHg) as was the proportion of animals receiving inotropes (NT_R: 9 (75%), NT_M: 8 (57%), and HT: 5 animals (50%)). In animals receiving inotropes, the median interquartile range (IQR) duration of dopamine administration was 1,135 min (818–1,219) in the NT_R group, 1,425 min (1,290–1,920) in the NT_M group and 1,208 min (850–1,576) in the HT group.

DISCUSSION

Minimal systemic HT combined with 48-h SHC following HI increased mortality and did not improve global neuropathological injury score in a newborn pig model. This is the first study of minimal HT combined with SHC in a preclinical survival model of perinatal asphyxia.

HT by 3–4 °C is neuroprotective after neonatal encephalopathy in term infants using SHC with moderate systemic HT or WBC (1). Minimal HT (36 °C) had a similar risk of death or poor neurologic function at 180 d compared with moderate HT (33 °C) in adults after cardiac arrest (16). SHC with minimal systemic HT may obviate the potential adverse effects of systemic HT such as cardiovascular compromise, pulmonary hypertension, infection, and coagulopathy, and be applicable to preterm infants.

Brain Cooling

Iwata *et al.* (17) found protection from intraischemic brain HT when T_{rectal} fell by 1 °C with 48-h survival in newborn pigs, but cap water temperatures of 0–10 °C caused significant scalp

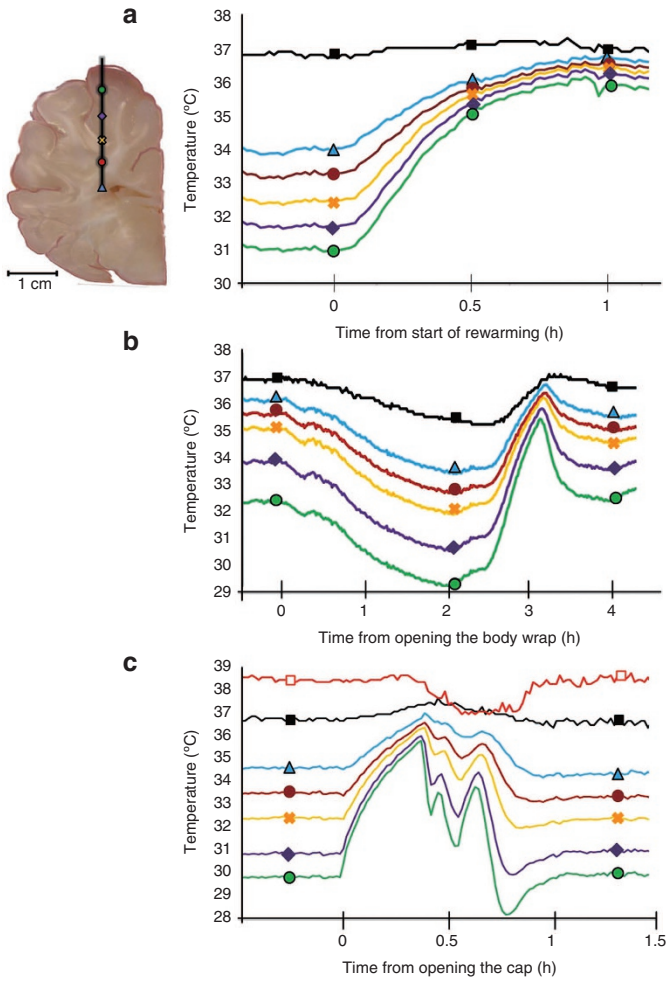

Figure 5. Brain, skin, and rectal temperature from three subjects. (**a**) T_{rectal} and T_{brain} during rewarming in a representative animal. Rewarming starts by discontinuing water flow through the cooling cap and continuing the overhead heating. This animal did not wear the water-perfused body wrap. There was a rapid increase in T_{brain} (5 mm (green, open circle), 10 mm (purple, diamond), 15 mm (orange, cross), 20 mm (brown, closed circle), and 25 mm (blue, triangle) depth from the brain surface) and loss of the gradient between T_{rectal} (black, square) and T_{brain}. Inset: coronal brain section illustrating the brain temperature probe position with sensors at 5-mm intervals. (**b**) Temperature changes in T_{rectal} and T_{brain} when the body wrap around the abdomen was opened to manage the umbilical lines. The body wrap heated the body to counteract the cooling effect of the cap (the way the CoolCap was designed to work), and opening it reduced body heating and hence T_{rectal} and T_{brain} were reduced. The cooling cap water temperature was initially kept the same. When the cooling cap water temperature was increased to prevent further decreases in T_{rectal} and T_{brain}, both temperatures rose rapidly with loss of the gradient between them. The gradient was previously maintained by cooling the brain and heating the body at the same time. (**c**) Temperature changes in T_{rectal}, T_{brain}, and T_{skin} when the cooling cap was opened. This subject received body heating from a combination of the body wrap and overhead heating. T_{rectal} increased and T_{brain} increased rapidly when the cooling cap was opened for skin inspection. The instructions for skin care while undergoing SHC with the Cool Cap instructs opening the cap to inspect the skin 12-hourly. This leads to a rapid increase in T_{brain} due to cessation of scalp cooling and slower increase in T_{rectal} due to unopposed heating from the body wrap. The additional overhead heating was discontinued to prevent further increase in T_{rectal} leading to a decrease in T_{skin} (red, open square). T_{rectal} and T_{brain} decreased when the cooling cap was replaced.

damage in all subjects. Tooley *et al.* (12) cooled $T_{deep\ brain}$ 7.7 °C lower than T_{rectal} for 8 h in newborn pigs but did not assess neuroprotection in a short-term survival study. We started treatment at the end of HI and continued for 48 h using the equipment currently used in clinical practice. We chose a treatment duration of 48 h instead of 24 h used previously in our model to compensate for cooling the brain at a higher temperature than during WBC.

Cooling the deep-brain structures using a cooling cap was limited by the heating capacity of the body wrap to counteract constant cooling from the cap because we challenged the equipment into a situation neither was originally designed for. In our previous much shorter study, we used cap temperatures around 7 °C cooler than the present study and SHC was balanced by radiant heaters in close proximity to the body, which resulted in high skin temperatures (40 °C) and tendency of hypotension that would be undesirable in clinical practice (12). The purpose-built cooling equipment for SHC and body warming was slow in achieving a balance between brain and body temperature.

Increased Mortality and No Neuroprotection

Despite lowering $T_{deep\ brain}$ by 3.5 °C (35.0 ± 1.1 °C) and $T_{superficial\ brain}$ by 7 °C (31.5 ± 1.6 °C) that would be expected to be neuroprotective from previous studies, we did not observe any benefit on global brain injury. Instead, we found increased mortality in cooled animals and only a trend toward less subcortical white matter injury. In all previous studies with HT under sedation, we used 6-, 12-, or 24-h HT and found neuroprotection (8,18,19) but we chose 48-h SHC in this study to counteract minimal systemic HT. However, 48-h cooling was not tolerated by those animals suffering severe insults trending toward lower blood pressure and longer duration of inotropic support. Similarly, in humans, 72-h HT is beneficial but a recent randomized study stopped cooling for 120 h because it was associated with high mortality (1,20). This has important implications for cooling outside trial protocols.

Cooling is more effective if starting early but our method takes 3–4 h to reduce brain temperature while maintaining T_{rectal} at 37 °C (21). The brain HT was obtained after 51 min when T_{rectal} was allowed to drop to 34.5 °C and after 40 min with WBC (8,18). HT is ineffective in our model if delayed by 3 h, and delayed HT is associated with decreased efficacy in other experimental and clinical studies (22–25).

Systemic inflammation and interplay between organs may be important in the propagation of brain injury (26), and WBC may antagonize cytokines or activate targets that attenuate brain injury (27). SHC combined with minimal HT may not attenuate the systemic inflammatory response as effectively as WBC, and inflammatory mediators may propagate brain injury despite selective brain cooling. In adult stroke patients, plasma levels of interleukin (IL)-6 predicted long-term outcome and WBC suppressed the IL-6 and IL-4 response in asphyxiated newborns compared with normothermic infants (28,29). Mobilization of the peripheral immune system and

Grade	Area affected (%)	Morphologic changes
1	≤10	Individual necrotic neurons, small patchy, complete, or incomplete infarcts
2	20–30	Partly confluent complete or incomplete infarcts
	40–60	Large confluent, complete infarcts
3	>75	In hippocampus: neuronal necrosis; in thalamus and basal ganglia: large complete infarcts; in cortex: total disintegration

Figure 6. Grading system for regional neuropathology. Reproduced from Table 1 in Thoresen *et al*. (15). "Incomplete" infarct describes a localized area where necrotic neurons are observed but other cell types including glia and vessels are preserved.

influx of neutrophils is observed in preterm sheep following HI, and the blood-brain barrier is more permeable in newborns particularly in the context of systemic inflammation and may allow the entry of circulating cytokines into the brain (30,31). The influence of systemic responses can be observed more easily using our global HI model than using a selective cerebral HI model.

Rapid Changes in Brain Temperature

The brain temperature increased rapidly to approach T_{rectal} during rewarming from SHC and also when removing the cap for skin inspection as instructed every 12 h (**Figure 3**). The rapid perfusion of cooled cerebral structures with warmer blood twice daily and at the end of treatment may be detrimental and certainly rapid rewarming of core temperature is associated with seizures (32). This has implications for current clinical practice of SHC where the cap is removed every 12 h and sharp rises in brain temperature may occur at least five times during treatment.

Cardiovascular Effects

The higher skin temperature and peripheral vasodilatation from body heating may explain why there was no net effect on cardiovascular status. This is consistent with SHC with moderate systemic HT in infants where the effect on inotropic support is uncertain (33). The mean HR decreased by 60 bpm during SHC equating to 17 bpm/°C using $T_{deep\ brain}$ or 40 bpm/°C using T_{rectal}, and we previously reported that HT reduces HR by around 10 bpm/°C. This suggests that brain temperature is a more important determinant for HR than body temperature, consistent with the temperature-sensing area being in the hypothalamus (34).

Study Limitations

The findings in preclinical animal models do not always translate successfully but previous work using this model has contributed to changes in clinical practice (8). It has been suggested that external cooling of the term human newborn head is only effective down to a depth of 2 cm from the scalp (5). We recorded a lowered brain temperature 2.2 cm from the brain surface in newborn pigs with head circumferences similar to preterm infants at 27-wk gestation. The differences between species may influence heat exchange and brain temperature. The pig brain is 2.3% of total body weight compared with around 15% in humans at term, which would make the former easier to cool although thinner skin and cranium may make it easier to cool the human brain (35,36).

Randomization to compare brain injury was unsuccessful due to selectively high mortality in cooled animals. In survivors, the duration of LAEEG during HI (an important measure of insult severity analyzed after the experiment) was shorter in the HT compared with the NT_R group, and we used regression analysis to assess treatment effect. In addition, we compared brain injury in HT animals with short insult duration with a matched group of NT controls that confirmed the findings of the first analysis. Thus, we compared HT with two different NT control groups to increase the validity of our conclusions.

In summary, we cooled the brain with only minimal body HT (1.5 °C below core temperature). SHC reduced $T_{superficial\ brain}$ by 7 °C from T_{rectal} and $T_{deep\ brain}$ by 2 °C. We found that 48-h minimal systemic HT combined with SHC was not protective and increased mortality following HI compared with systemic NT. It took ~4 h to cool the brain with this method.

METHODS

Experiments were carried out under a Home Office licence and approved by the University of Bristol Ethical Review Panel.

Preparation

Crossbred Landrace pigs were born at term gestation (113–115 d). Experiments were started the first day of life at 16 ± 5 h mean ± SD postnatal age. Pigs were anesthetized (2% isoflurane, 33% O_2, and 65% N_2O), intubated, and ventilated (SLE 2000; SLE, Surrey, UK, with 1–2% isoflurane, 70% N_2O, and 28–29% O_2) using end-tidal CO_2, transcutaneous O_2 saturation, and arterial blood gases analyzed at 37 °C (i-STAT, Abbott, Birmingham, UK; RapidLab 248 pH/blood gas analyzer, Siemens Healthcare Diagnostics, Surrey, UK) to maintain pH, PCO_2, and PO_2 in the normal range (pH 7.35–7.45, PCO_2 4.5–6 kPa, and PO_2 10–13 kPa). The MABP was monitored using an umbilical catheter. A T_{rectal} probe (reusable YSI 400 series, Criticool, MTRE, Yavne, Israel) was inserted to 6 cm, and a skin temperature probe (Criticool) was sited on a hind limb and animals were maintained at NT for pigs (T_{rectal} 38.5 °C) using a water-circulated body wrap and temperature unit (Criticool) servo-controlled to T_{rectal} or a radiant heater. An artificial fontanelle and a burr hole were created in the skull to insert a probe (Thermes, Physitemp Instruments, Clifton, NJ) with multiple sensors measuring cortical (5 mm from the brain surface) and deep gray matter/basal ganglia (25 mm from the cortex) temperature. Three or five needle electrodes (019-409700 0.4 mm (27G) Viasys Healthcare, Chicago, IL) were inserted subdermally into the scalp with an interelectrode distance of 3 cm to monitor amplitude-integrated EEG (aEEG/EEG) (aEEG, EEG) (Olympic or Brainz, Natus Medical Incorporated, San Carlos, CA). The gas mixture was changed to 0.7% halothane, 29.3% O_2, and 70% nitrogen 30 min before HI.

Hypoxic-Ischemic Insult

As previously described inspired O_2 was reduced to ~6% for 45 min, causing depression of the background aEEG activity to below 7 μV (LAEEG) (18). The duration of LAEEG out of the total 45 min correlates with the outcome-severity of the insult (15). The animals were resuscitated in air and anesthesia continued using propofol (4–12 mg/kg/h) and remifentanil (20–80 mcg/kg/h).

Randomization and Inclusion in the Study

The animals were randomized immediately after the 45-min insult to NT_R (T_{rectal} 38.5 ± 0.2 °C, n = 12) or HT (T_{rectal} 37.0 ± 0.2 °C for 48 h,

$n = 14$) combined with SHC followed by 24-h NT. The animals needed to survive at least 48 h, which is the treatment period to be included.

Matched Normothermic Treatment Group

A valid neuropathology assessment was not available for all animals because of significantly more early deaths (before the end of the 48-h treatment period) of 43% in the HT compared with 17% in the NT_R group. The total duration of LAEEG was shorter in the HT than the NT_R group when animals without pathology score were not included and insult severity was not comparable. Therefore, the HT group was matched with a group of historical controls (NT_M) using the total duration of LAEEG during HI and arterial pH at the end of HI, which are the two best predictors of brain injury in this model (15). There were 10 animals in the HT group matched with 14 in the NT_M group that underwent similar preparation, HI, and intensive care apart from temperature control.

Intensive Care

Care followed protocols as described previously including maintenance fluids (5% dextrose/0.45% saline 12 ml/kg/h) and antibiotics (amoxicillin 30 mg/kg/12 h, gentamicin 5 mg/kg/24 h) (18). Hypotension (MABP < 40 mmHg for ≥10 min) was managed with fluid boluses and inotropes in a stepwise manner. Clinical and electrical seizures on continuous EEG/aEEG recordings lasting >5 min were treated with anticonvulsants. The seizures were identified on aEEG by an abrupt increase in minimum and maximum amplitude (>10 μV) lasting >20 s and on EEG by gradual build-up and then decline in frequency and amplitude of repetitive spikes or sharp wave activity. The target blood glucose was 3–8 mmol/l and hypoglycemia was managed with a 10% dextrose 2.5 mls/kg bolus and increased glucose delivery up to 12 mg/kg/min.

Temperature Control

T_{rectal} was maintained at 38.5 °C in the NT group using a servo-controlled water-circulated body wrap (Criticool) or a manually adjusted radiant warmer. In the HT group, T_{rectal} was reduced to 37.0 °C at the end of HI. SHC was achieved using a water-circulated cap used clinically consisting of a network of channels holding 75 ml of water that was applied to the head and snout (Cool Care System Olympic Medical, Seattle, WA) (37). A disposable nappy covered the cap as an insulating layer with a reflective shield (Olympic Medical) covering the head and neck. Water circulated through the cap at 750 ml/min between 5 and 30 °C controlled by a mobile cooling unit (Cool Care System). The temperature of water was gradually decreased from 30 °C to a median (IQR) of 18 °C (15–21) to maintain a gradient between brain and body with stable target T_{rectal}. The body wrap warmed the body and additional overhead heating was used as fine-tuning. SHC and relative body warming continued for 48 h before rewarming was commenced by stopping water flow through the cap and removing after 1 h. T_{rectal} was increased from 37.0 °C to 38.5 °C over 4.5 h at 0.3 °C/h. The animals were maintained at T_{rectal} of 38.5 °C using the servo-controlled body wrap for further 24 h until kill at 72 h after HI.

Pathology

The animals were killed after 72-h survival and brains were perfusion-fixed (0.9% NaCl and 10% phosphate-buffered formalin) under deep anesthesia. The right (uninstrumented) cerebral hemisphere was blocked at 5-mm intervals, paraffin-embedded, and 6-μm sections from 13 blocks stained with hematoxylin and eosin. A perinatal pathologist who was blinded to treatment and the clinical course assessed brain injury in six regions (cortical gray matter, subcortical white matter, basal ganglia, thalamus, hippocampus, and cerebellum) using a nine-point score from 0.0 to 4.0 with intervals of 0.5, and a global score was calculated from the mean of regional scores (**Figure 6**) (15).

Statistical Methods

Mean (SD) and ANOVA with Bonferonni posthoc test were used for normally distributed continuous data and median (IQR) and the Kruskal–Wallis test was used when data did not have a normal distribution with $P < 0.05$ (two-tailed testing) considered significant (IBM SPSS Statistics v21.0, Armonk, NY). The stepwise linear regression analysis was performed with global neuropathology score as the dependent variable using a significance value of 0.05 when allowing independent variables into the regression model.

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