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

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INTRODUCTION: The precise temperature for optimal neuroprotection in infants with neonatal encephalopathy is unclear. Our aim was to assess systemic effects of whole-body cooling to 35 °C, 33.5 °C, and 30 °C in a piglet model of perinatal asphyxia.

METHODS: Twenty–eight anesthetized male piglets aged <24 h underwent hypoxia–ischemia (HI) and were randomized to normothermia or cooling to rectal temperature (Trec) 35 °C, 33.5 °C, or 30 °C during 2–26 h after insult ($n = 7$ in each group). HR, MABP, and Trec were recorded continuously.

RESULTS: Five animals cooled to 30 °C had fatal cardiac arrests. During 30 °C cooling, heart rate (HR) was lower vs. normothermia ($P < 0.001$). Although mean arterial blood pressure (MABP) did not vary between groups, more fluid boluses were needed at 30 °C than at normothermia ($P < 0.02$); dopamine use was higher at 30 °C than at normothermia or 35 °C ($P = 0.005$ and $P = 0.02$, respectively). Base deficit was increased at 30 °C at 12, 24, and 36 h vs. all other groups ($P < 0.05$), pH was acidotic at 36 h vs. normothermia ($P = 0.04$), and blood glucose was higher for the 30 °C group at 12 h vs. the normothermia and 35 °C groups ($P < 0.05$). Potassium was lower at 12 h in the 30 °C group vs. the 33.5 °C and 35 °C groups. There was no difference in cortisol level between groups.

DISCUSSION: Cooling to 30 °C led to metabolic derangement and more cardiac arrests and deaths than cooling to 33.5 °C or 35 °C. Inadvertent overcooling should be avoided.

Therapeutic hypothermia is established as a safe and effective treatment for moderate to severe neonatal encephalopathy in the developed world (1); its clinical introduction followed decades of carefully conducted preclinical studies (2) and clinical trials (3–7). In these trials, which included whole-body (3–5) and selective head (6,7) cooling, the target core target temperature was 33–35 °C (2–4 °C below normothermia).

There is little information defining the optimal temperature for cooling following a hypoxic–ischemic (HI) insult. Cooling to both 32 °C and 34 °C reduces impairments (histological and behavioral) assessed 6 mo after brief forebrain ischemia in the gerbil (2). In a small study of fetal sheep, with cooling starting 90 min after HI, protection was seen only with a sustained extradural temperature ≤34 °C (8) and in the piglet, 35 °C provided better neuroprotection in deep gray matter than 33 °C (9). However, in adult primates 48-h cooling at 29 °C following middle-cerebral artery occlusion was associated with worse outcomes (100% fatality) as compared with normothermia (10). Systemic adverse effects of hypothermia appear to be proportional to the drop in temperature (11), with most adverse effects occurring at core temperatures below 34 °C.

In a recent meta-analysis in which studies were grouped into those with a target core temperature >34 °C or those with a target core temperature ≤34 °C, there was no difference in the relative risk of any neurodevelopmental outcome among patients who received hypothermia with target core temperature >34 °C as compared to the control group. Therefore, the optimal temperature for neural rescue is likely to lie at some point below 34 °C (12); however, a threshold temperature must exist below this where the adverse systemic effects of cooling outweigh the potential neurological benefits of cooling. This threshold temperature may vary with the type or severity of injury as well as with intrinsic local tissue susceptibility.

The developed world has now adopted therapeutic hypothermia into clinical practice (3,13). There have been reassuring studies describing the safety of therapeutic hypothermia in asphyxiated newborns (12,14). It is important to appreciate that these safety reports are based on cooling administration under intensive care with strict cooling protocols at experienced centers. However, episodes of excessive drops in temperature are increasingly reported, especially in the absence of continuous core temperature monitoring. One study reported at least one recorded temperature <30 °C and <32 °C in 14% and 34%, respectively, of infants undergoing active cooling during transport to a cooling center (15); other pilot studies have intentionally targeted core temperatures of 32.2 °C (±0.9) for 72 h (16).

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The aim of this study was to assess the systemic effects of cooling for 24 h at 35 °C, 33.5 °C, and 30 °C in a piglet model of perinatal asphyxia. This was part of a larger study assessing the optimal temperature for neuroprotection (reported elsewhere).

RESULTS

There were no intergroup differences in body weight, postnatal age, insult severity, or baseline physiological and biochemical measures (Table 1). Six piglets cooled to 30 °C suffered an episode of cardiac arrest, and five of these died before 48-h post-HI because of a combination of pulmonary edema, fluid in the thorax or abdominal cavities, severe metabolic derangement, or profound hypotension. One animal cooled to 33.5 °C suffered two cardiac arrests but survived to 48 h. There was one recorded incidence of persistent arrhythmia in the 30 °C group.

Physiological Measures

During cooling induction and rewarming/normothermia, mean heart rate (HR) and mean arterial blood pressure (MABP) were similar in all groups (Table 1). Baseline HR averaged over all groups was 154 (17) beats per minute (bpm); in the 33.5 °C and 35 °C groups during hypothermia, HR was unchanged but in the 30 °C group, HR was lower during hypothermia than in the normothermic piglets ($P < 0.001$, Figure 1a).

Several animals cooled to 30 °C experienced periods of profound hypotension (~30 mm Hg) lasting several hours; these episodes usually preceded cardiac arrest and were mainly experienced by the five animals in the 30 °C group that died before 48 h. However, when averaged over each analysis period, the MABP was similar to baseline in all of the cooling groups (Figure 1b).

Volume replacement was required in two normothermic animals following HI; these animals required no inotropes. The overall median volume replacement (saline and Gelofusin) was higher for the 30 °C group as compared with the normothermia group ($P = 0.05$; Kruskal–Wallis and Mann–Whitney tests) (Table 2). The median dopamine infusion dose over 48 h was higher for the 30 °C group than for the normothermia ($P = 0.01$) or 35 °C ($P = 0.05$, Kruskal–Wallis and Mann–Whitney tests) (Table 2) groups. The median dopamine infusion was also greater for the 33.5 °C group than for the normothermia group ($P = 0.05$). In addition, multiple inotrope infusions (dopamine, dobutamine, and adrenaline) were required during maintenance and rewarming/normothermia in the 30 °C group, whereas only dopamine was required in the other groups.

Table 1. Physiological variables for piglets in each temperature group

Variable	Normothermia	35 °C	33.5 °C	30 °C
Postnatal age, h	22.3 (1.2)	22.6 (1.1)	22.7 (0.9)	22.5 (1.1)
Body weight, g	1,771 (132)	1,786 (90)	1,714 (146)	1,864 (180)
AED (insult severity), h	0.07 (0.04)	0.05 (0.03)	0.06 (0.04)	0.08 (0.07)
HR, bpm				
Baseline	158 (26)	162 (32)	152 (28)	144 (21)
End of insult	182 (28)	184 (30)	187 (32)	172 (29)
2–3.5 h after time zero	155 (28)	130 (26)	132 (30)	130 (11)
3.5–26 h after time zero	156 (15)	121 (28)	114 (21)	123 (16)*
26–48 h after time zero	151 (22)	146 (33)	140 (17)	149 (4)
MABP (mm Hg)				
Baseline	51 (6)	53 (7)	53 (9)	51 (6)
End of insult	51 (10)	59 (16)	58 (11)	55 (6)
2–3.5 h after time zero	45 (6)	43 (5)	44 (8)	47 (7)
3.5–26 h after time zero	51 (6)	48 (6)	47 (6)	43 (7)
26–48 h after time zero	52 (6)	51 (7)	47 (6)	48 (11)
T rectal (°C)				
Baseline	38.4 (0.4)	38.2 (0.6)	38.1 (0.8)	38.4 (0.4)
End of insult	38.2 (0. 4)	38.1 (0.3)	38.1 (0.4)	38.2 (0.4)
6–26 h after time zero	38.5 (0.4)	34.9 (0.4)*,‡	33.2 (0.5)*,**,‡	30.2 (0.4)*,**,†,‡
26–48 h after time zero	38.4 (1.3)	37.7 (1.2)	37.0 (1.8)*,**,‡	34.4 (2.6)*,**,†,‡

Mean (SD) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and *post hoc* analysis were carried out on comparisons between groups with Tukey's and Dunett's method.

Time zero was set at the start of resuscitation after the hypoxic–ischemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 min of resuscitation.

AED, acute energy depletion; HI, hypoxia–ischemia; HR, heart rate; MABP, mean arterial blood pressure; T rectal, rectal temperature.

*$P < 0.05$ vs. normothermia at the same time point or during the same time period. **$P < 0.05$ vs. 35 °C at the same time point or during the same time period. †$P < 0.05$ vs. 33.5 °C at the same time point or during the same time period. ‡$P < 0.01$ in cross-group comparisons.

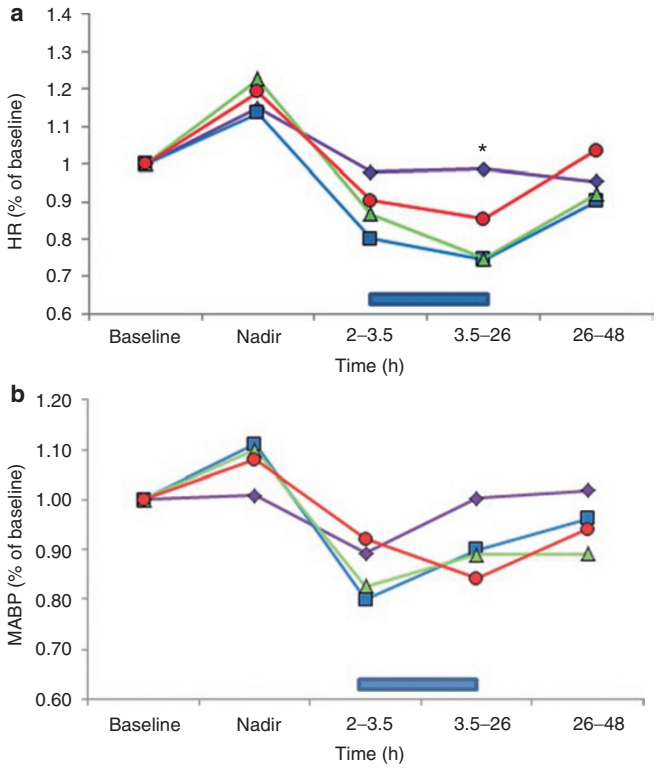

Figure 1. Mean % change in (**a**) heart rate (HR) and (**b**) mean arterial blood pressure (MABP) during and after 24-h whole-body hypothermia for each temperature group: 38.5 °C (purple diamonds), 35 °C (blue squares), 33.5 °C (green triangles), and 30 °C (red circles) following transient hypoxia–ischemia. The blue bars represent the duration of the cooling period. *Significant difference between the 30 °C and the normothermia (38.5 °C) groups ($P < 0.001$). Nadir, hypoxia–ischemia midpoint; Time 0 h, end of hypoxia–ischemia.

Table 2. Median (IQR) total volume replacement (ml/kg) and inotrope dose (μg/kg/min) over the 48-h period following hypoxia–ischemia according to each temperature group

	Normothermia	35 °C	33.5 °C	30 °C
Volume replacement (ml/kg)	15 (0, 31)	19 (0, 38)	18 (0, 82)	70 (46-108)*
Inotropes (μg·kg·min)				
Dopamine	0 (0, 0)	0 (0, 4.8)	5.5 (0.7, 11.1)*	13.8 (8.2, 18.6)*,**
Dobutamine	0 (0, 0)	0 (0, 0)	0 (0, 0)	16.0 (0, 18.7)
Noradrenaline	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.1 (0, 0.3)

Kruskal–Wallis equality-of-populations rank test ($P < 0.05$). Where no inotropes were given a "0" value was assumed.

IQR, interquartile range.

*$P < 0.05$ vs. normothermia at the same time point or during the same time period.
**$P < 0.05$ vs. 35 °C at the same time point or during the same time period.

Blood Biochemistry

Base deficit was increased in the 30 °C group at 12, 24, and 36 h as compared with the other groups (all $P < 0.05$; Table 3 and Figure 2a); base deficit was similar among all other groups at these times. Blood pH was lower in the 30 °C group at 36 h ($P = 0.05$), with borderline *post hoc* significance as compared with the normothermic group at 12 h ($P = 0.05$, Table 3 and Figure 2b).

In the 30 °C group, blood glucose was higher at 12 h than that of both the normothermic and 35 °C groups ($P = 0.05$ and $P = 0.05$, respectively) but had normalized by 24 h (Table 3 and Figure 3a,b). Blood lactate was highest at 24 h in the 30 °C group but was not significantly different from the other groups (Table 3 and Figure 3c,d). Blood potassium was lower in the 30 °C group at 12 h as compared with the 35 °C and 33.5 °C groups ($P = 0.01$ and $P = 0.05$, respectively). Hematocrit was greater in the 30 °C group at 24 h as compared with the normothermia and 33.5 °C groups (both $P = 0.05$), and hemoglobin was greater in 30 °C group at 12 h as compared with the 35 °C group ($P = 0.05$) and at 24 h as compared with the 33.5 °C group ($P = 0.05$) (Table 4).

Shivering episodes (nonseizure related) were no different between groups. There was no difference between groups in serum cortisol at any time point, although there was a suggestion that cortisol was increased 2 h following HI and reduced at 12 h (during the cooling phase) after HI (Figure 4).

Mean cardiac troponin-I levels were significantly lower in the 30 °C group as compared with all other groups over 12–48 h following HI ($P \leq 0.01$, Figure 5).

Macroscopic Organ Pathology

Macroscopic organ pathology was noted in 25%, 33%, 50%, and 33% of individuals in the 38.5 °C, 35 °C, 33.5 °C, and 30 °C groups, respectively (Table 5). Patchy sinusoidal congestion of the liver was a common finding across all groups. Evidence of pneumonia and severe pneumonia occurred in all but the 30 °C group and vacuolated kidney in all but normothermic group. Incidences of acute tubular necrosis and liver steatosis were limited to the 33.5 °C and 30 °C groups only. No evidence of macroscopic pathology was noted in two hearts (30 °C and 33.5 °C). No organ pathology was noted in two naive piglets.

DISCUSSION

In our piglet model of perinatal asphyxia, we demonstrated abnormal metabolic homeostasis (lactic acidosis, hyperglycemia, and hypokalemia), increased need for inotrope and fluid bolus support to maintain MABP, and more fatalities with 30 °C cooling as compared with normothermia or cooling to 35 °C or 33.5 °C. These results have relevance for the use of hypothermia in clinical practice and emphasize the importance of strict adherence to the target therapeutic temperature and the potential dangers of inadvertent overcooling below the target range of 33–34 °C ("hypothermic overshoot"). Although no hypothermia clinical trial has targeted core temperatures as low as 8 °C below core temperature, inadvertent overcooling can occasionally occur with passive cooling as well as servo-controlled and non-servo-controlled cooling devices (18,19). These data are similar to those from the first hypothermia clinical trials in the 1960s (20), which used deep hypothermia (30 °C); these trials were discontinued because of side effects

Table 3. Blood gas variables for piglets in each temperature group

Variable	Normothermia	35 °C	33.5 °C	30 °C
PaO_2				
Baseline	9.3 (1.1)[§§]	10.5 (1.7)	9.4 (1.9)	8.3 (1.7)
Nadir of the insult	3.3 (1.0)[§,§§]	3.1 (1.1)[§]	3.1 (1.2)	3.0 (1.0)[§]
12 h after time zero	10.3 (1.2)	8.8 (1.2)	9.2 (5.3)	8.0 (2.9)
24 h after time zero	11.2 (2.6)[§]	12.3 (6.3)	9.8 (3.3)	7.5 (2.2)
36 h after time zero	10.9 (2.2)	10.0 (3.6)	11.5 (4.5)	8.9 (1.7)
48 h after time zero	11.7 (2.2)	11.6 (3.0)	14.2 (6.3)	11.7 (1.9)
$PaCO_2$				
Baseline	5.46 (0.74)	5.26 (1.05)	5.26 (0.65)	4.98 (0.76)
Nadir of the insult	4.36 (0.49)	4.88 (1.22)	5.27 (0.94)	5.02 (0.82)
12 h after time zero	5.29 (0.93)	4.81 (0.71)	6.17 (0.86)	5.83 (2.13)
24 h after time zero	4.23 (1.03)	6.08 (1.71)	4.96 (1.34)	4.82 (2.43)
36 h after time zero	5.29 (1.11)	5.68 (1.36)	6.80 (1.26)	6.82 (1.35)
48 h after time zero	5.21 (1.28)	5.15 (0.71)	6.15 (1.43)	4.47 (0.51)
pH				
Baseline	7.49 (0.04)	7.50 (0.09)	7.52 (0.06)	7.51 (0.07)
Nadir of the insult	7.47 (0.09)	7.44 (0.05)	7.43 (0.16)	7.44 (0.08)
12 h after time zero	7.57 (0.08)	7.54 (0.09)	7.45 (0.05)	7.37 (0.20)
24 h after time zero	7.59 (0.09)	7.45 (0.14)	7.48 (0.14)	7.37 (0.28)
36 h after time zero	7.47 (0.10)	7.45 (0.10)	7.37 (0.10)	7.29 (0.11)*
48 h after time zero	7.45 (0.11)[§§]	7.47 (0.07)	7.38 (0.09)	7.52 (0.09)
Base deficit (mmol/l)				
Baseline	8.2 (3.6)	7.8 (2.1)	9.6 (5.4)	7.5 (4.5)
Nadir of the insult	0.7 (5.4) [§,§§]	0.0 (4.0)[§,§§]	2.2 (8.0)	1.2 (3.0)
12 h after time zero	11.7 (3.3)	9.7 (1.0)	9.3 (2.7)	0.6 (8.8)*,**,†,‡
24 h after time zero	8.5 (3.7)	7.2 (2.6)	3.6 (5.5)	−5.5 (8.8)*,**,†,‡
36 h after time zero	6.1 (3.0)	5.6 (1.5)	3.6 (3.8)	−3.1 (6.0)*,**,†
48 h after time zero	3.6 (4.0)	5.0 (2.6)	2.1 (4.0)	4.5 (5.4)
Lactate (mmol/l; median (IQR))				
Baseline	3.4 (2.6, 4.3)	3.7 (2.9, 4.0) f	2.7 (2.4, 5.5)	3.3 (2.8, 3.8)
Nadir of the insult	7.6 (6.47, 8.5)[§,§§]	7.4 (6.7, 8.4)[§,§§]	6.9 (6.0, 9.7)[§,§§]	8.2 (6.7, 9.0)
12 h after time zero	1.8 (1.3. 1.9)	1.6 (1.1, 1.9)[§]	1.3 (0.7, 1.7)	2.2 (1.7, 10.0)
24 h after time zero	2.4 (1.6, 3.0)	1.6 (0.9, 1.7)[§]	1.8 (1.5, 3.3)	3.6 (2.1, 12.0)
36 h after time zero	1.0 (0.8, 1.7)	1.5 (1.5, 1.6)	1.1 (1.0, 1.4)	2.7 (1.5, 3.8)
48 h after time zero	1.3 (1.2, 1.4)	1.3 (1.1, 2.1)[§]	1.2 (1.1, 1.8)	2.6 (1.8, 3.2)
Glucose (mmol/l)				
Baseline	7.5 (6.1, 8.1)[§§]	6.8 (6.1, 7.5)	6.2 (5.7, 6.5)	7.6 (6.9, 8.3)[§§]
Nadir of the insult	9.8 (8.3, 10.4)[§,§§]	7.8 (7.7, 8.9)	8.4 (7.5, 9.8)	8.5 (8.5, 9.5)
12 h after time zero	5.2 (5.0, 6.2)[§]	5.5 (4.4, 7.0)	9.3 (4.7, 10.7)	17.2 (9.7, 28.7)*,**,[§]
24 h after time zero	5.4 (4.8, 6.2)[§]	5.4 (4.5, 9.6)	5.5 (5.1, 8.5)	23.9 (12.2, 25.9)[§]
36 h after time zero	4.9 (4.1, 6.2)	6.4 (6.1, 7.4)	5.5 (4.3, 11.6)	15.9 (4.8, 15.9)
48 h after time zero	4.8 (3.9, 5.2)	4.9 (4.7, 5.2)	4.3 (3.0, 5.5)	5.8 (4.3, 6.4)[§§]

Mean (SD) or median (interquartile range (IQR)) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and *post hoc* analysis were carried out on comparisons between groups with Tukey's and Dunett's method. Time zero was set at the start of resuscitation after the hypoxic–ischemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 min of resuscitation.

HI, hypoxia–ischemia; $PaCO_2$, partial arterial pressure of carbon dioxide; PaO_2, partial pressure arterial of oxygen.

*$P < 0.05$ vs. normothermia at the same time point or during the same time period. **$P < 0.05$ vs. 35 °C at the same time point or during the same time period. †$P < 0.05$ vs. 33.5 °C at the same time point or during the same time period. ‡$P < 0.01$ in cross-group comparisons. §$P < 0.05$ for within-group comparisons vs. baseline. §§$P < 0.05$ for within-group comparisons vs. 24 h after time zero.

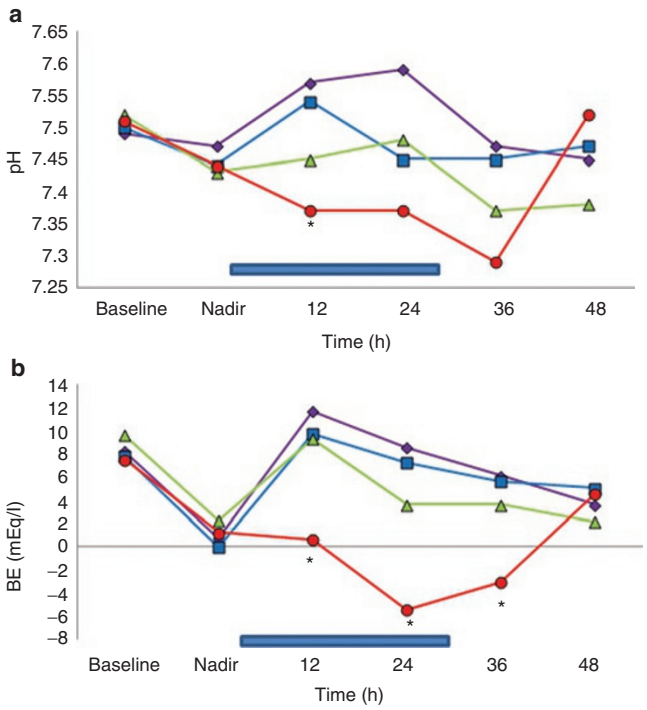

Figure 2. Mean (**a**) base excess (BE) and (**b**) blood pH at baseline, nadir, and 12, 24, and 48 h post-HI for each temperature group: 38.5 °C (purple diamonds), 35 °C (blue squares), 33.5 °C (green triangles), and 30 °C (red circles). The blue bars represent the duration of the cooling period. *Significant difference between the 30 °C group and all other groups ($P < 0.05$) at 12, 24, and 36 h. Nadir, hypoxia–ischemia midpoint; Time 0 h, end of hypoxia–ischemia. HI, hypoxia–ischemia.

and uncertain benefit. Interest was rekindled in the early 1990s, when animal studies showed mild hypothermia (32–35 °C) was beneficial and had fewer side effects than deep cooling (2,21).

Hypothermia reduces metabolic rate by 7–9% per 1 °C core temperature reduction with parallel decreases in oxygen consumption and CO_2 production (22); other mechanisms of hypothermic neuroprotection include the following: reduced excitotoxicity, calcium antagonism, protein synthesis preservation, decreased edema, modulation of the inflammatory cascade, and a change in pro- and anti-apoptotic signaling (23).

Cardiovascular responses to moderate hypothermia include peripheral vasoconstriction, sinus bradycardia with prolonged QT interval, reduced HR, and a decrease in cardiac output and ejection fraction (12,24); deep hypothermia (<30 °C) decreases myocardial contractility (25). Clinically benign sinus bradycardia was the only significant cardiovascular effect of hypothermia reported in all recent cooling trials (12,14). In our current study, we observed sinus bradycardia in the 30 °C group. More piglets in the 30 °C cooling group died as a result of cardiac arrest and fatal arrhythmias, similar to results described in other models using moderate to profound cooling (10,24). The arrhythmia threshold was ~31 °C; as core temperature fell below 30 °C, ventricular fibrillation became more likely.

In our study, animals cooled to 30 °C (8.5 °C drop in core temperature) required greater volume and inotropic support to maintain MABP. Animals cooled to 33.5 °C required more dopamine than the normothermia and 35 °C groups, whereas animals cooled to 35 °C had no increased requirements in volume or inotropes as compared with the normothermia group. In clinical cooling trials (3–4 °C drop in core temperature), hypotension was not seen more frequently with cooling; inotrope use was "physician related" with a slower withdrawal of therapy in cooled infants than noncooled infants (26).

Pilot clinical studies and clinical trials of cooling to a core temperature of 33–34 °C have shown no physiological or clinical differences with mild cooling apart from higher incidences of arrhythmias and thrombocytopenia (12). Azzopardi *et al.* (17) observed mild hypokalemia in a pilot cooling study; hypothermia-induced hypokalemia has been seen commonly in experimental and clinical studies (27) and relates to the intracellular shift in potassium, increased sympathetic tone, and stimulation of B2-adrenergic receptors (28). In piglets cooled to 30 °C, we observed hypokalemia at 12 h. Increased hematocrit was seen in the 30 °C group; this was unexpected in light of the large volume of fluid administered to maintain MABP. Hyperglycemia was also observed in the 30 °C group; this may be secondary to an adreno-sympathetic response from hypoperfusion. This combined with reduced oxygen delivery to tissues due to a shift in the hemoglobin–oxygen dissociation curve would further enhance peripheral vasoconstriction, lactic acidemia, and hypovolemia. Hyperglycemia produces enhanced cerebral injury in adult humans and animal models of HI (29), although in neonatal models the influence of hyperglycemia on brain injury is more varied; several studies demonstrate a beneficial effect of glucose administration prior to or immediately after cerebral ischemia (30) with phosphocreatine and adenosine triphosphate preservation (31).

We did not use muscle relaxants in this model, and sedation and anesthesia were administered at all times. A previous study demonstrated that cooling in unsedated piglets results in increased cortisol levels, shivering, and loss of hypothermic neuroprotection (32). However, in our study, shivering frequency and serum cortisol were no different between groups at any time point. Cardiac troponin-I levels were lower in the 30 °C cooling group between 12 and 48 h as compared with all other groups in our study. Cardiac troponin-I is a robust marker of cardiomyocyte injury and is used as a biomarker with a high sensitivity to diagnose myocardial cell injury in adult cardiology (33). Our data concur with the recent findings in another piglet model of asphyxia in which cooling to 34.5 °C after the injury reduced serum cardiac troponin levels at 6 h as compared with normothermia (34). In our study, we show that cooling to 30 °C reduced cardiac troponin even further than 33.5 °C and 35 °C, suggesting that deeper cooling temperatures could be more protective to cardiac muscle.

Organ pathology was noted in all groups on macroscopic examination (**Table 5**). Acute tubular necrosis was noted in 33.5 °C and 30 °C groups. It is likely that excessive cooling was more detrimental in subjects with preexisting severe multiorgan damage. The threshold minimum safe cooling temperature

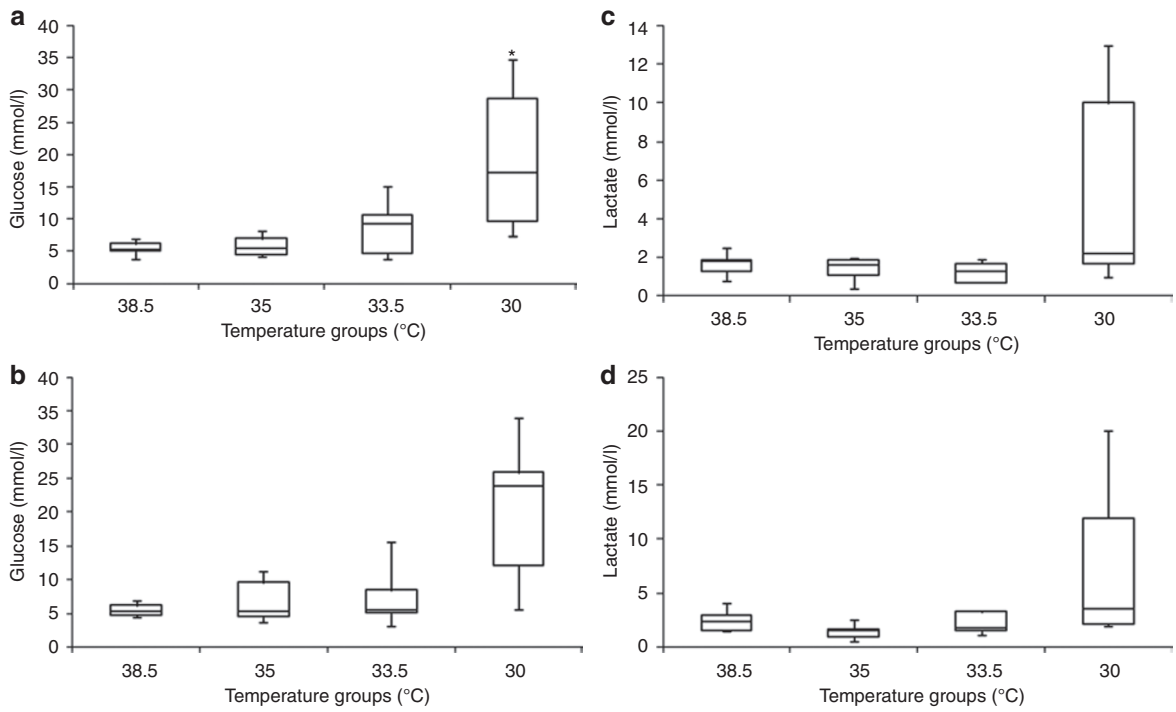

Figure 3. Levels of median (interquartile range) (**a**,**b**) blood glucose and (**c**,**d**) lactate at (**a**,**c**) 12 and (**b**,**d**) 24 h post-HI. *Significant difference between the 30 °C group and the normothermia and 35 °C groups for glucose at 12 h only ($P < 0.05$). HI, hypoxia–ischemia.

may therefore depend on brain injury severity and hypoxic–ischemic organ dysfunction.

In clinical practice, it is important to differentiate accidental from therapeutic hypothermia. The World Health Organization reported global neonatal mortality rates of 39%, 52%, and 80%, respectively, with mild, moderate, and severe hypothermia at hospital presentation (35). We know from the clinical cooling trials in high-resource settings that mild therapeutic hypothermia (33–35 °C) is safe under intensive care conditions in tertiary centers that carefully control core temperatures and maintain MABP and metabolic homeostasis. However, our results suggest that overcooling asphyxiated infants may be deleterious even in high-resource settings if metabolic homeostasis cannot be maintained.

Meta-analysis (1) of three large pragmatic trials (3,5,6) showed that therapeutic hypothermia reduces death or disability at 18 mo with a risk ratio of 0.81 (95% confidence interval (CI) 0.71–0.93) and a number needed to treat of 9 (95% CI 5–25). Some researchers question whether deeper and longer cooling might benefit more infants. A few asphyxiated infants have been cooled to a core temperature of 32.2 °C (± 0.9) for 72 h in a pilot study (16). A large trial is in progress evaluating deeper and longer cooling periods (32 °C for 120 h vs. 33.5 °C for 72 h) for infants presenting at age <6 h (http://clinicaltrials.gov/ct2/show/results/NCT01192776). Until the results of this trial are known, it is important to ensure that overcooling does not occur and that the target range of 33–34 °C is maintained during cooling.

There are some limitations to our study. Some species (such as the dog and piglet) may be more sensitive to the systemic side effects of deep hypothermia than humans. In addition, as compared with the human infant, the relative decrease in temperature with cooling to 30 °C, 33.5 °C, and 35 °C is greater in the piglet (maximum drop in core temperature: 8.5 °C at 30 °C); such temperature reductions thus incur a comparatively larger metabolic-rate reduction. We initiated cooling ~2 h after HI and maintained it for 24 h. Our hypothermia initiation delay is shorter than the median time of recruitment for the randomized clinical trials reported so far (3–6) but is in keeping with the evolving practice of earlier cooling and passive cooling during transport to a cooling center. In addition, our cooling duration is shorter than current cooling protocols (72 h); however, 24-h cooling offers comparable neuroprotective efficacy in the piglet and is practical and feasible. Following HI, we do not generally observe lactic acidosis in the piglet model; this differs from the persisting lactic acidosis seen in some encephalopathic infants in the acute phase following a prolonged resuscitation. The difference may relate to the nature of the localized ischemic and global hypoxic insult in the piglet model of asphyxia.

The anesthesia used in our model (isoflurane 1–4%) constitutes a difference between experimental and clinical protocols. Gaseous anesthetics decrease cardiac output, stroke volume, and ejection fraction by ~75% from conscious levels (36). We cannot exclude an interaction between different degrees of hypothermia and isoflurane that may have potentiated effects on cardiac rhythm and contractility. Fentanyl without isoflurane may have resulted in less negative hemodynamic effects; however, this cannot be explored in our preclinical model. Finally, we used pH-stat for acid–base management because in preclinical piglet (37) and clinical studies (38), neurologic recovery was improved with pH-stat as compared with α-stat

Table 4. Blood chemistry for piglets in each temperature group

Variable	Normothermia	35 °C	33.5 °C	30 °C
Sodium (mmol/l)				
Baseline	133.4 (3.4)	131.3 (3.6)	130.2 (2.6)	130.7 (2.9)
Nadir of the insult	132.0 (3.6)	131.3 (4.7)	130.2 (3.7)	130.8 (3.5)
12 h after time zero	128.3 (3.2)	125.7 (3.6)	126.3 (2.7)	128.7 (8.5)
24 h after time zero	127.9 (3.0)	124.3 (3.6)	125.0 (3.7)	124.6 (6.9)
36 h after time zero	128.4 (6.9)	122.8 (5.7)	125.4 (2.2)	127.1 (3.3)
48 h after time zero	128.1 (8.0)	125.8 (1.9)	126.1 (4.5)	130.3 (3.6)
Potassium (mmol/l)				
Baseline	3.76 (0.69)	4.49 (1.11)	4.96 (0.54)	3.87 (1.42)
Nadir of the insult	3.92 (0.82)	4.55 (1.15)	5.22 (1.07)	3.68 (1.10)
12 h after time zero	4.95 (0.77)	5.76 (0.71)	5.25 (0.77)	3.89 (0.92)**,†
24 h after time zero	4.77 (0.89)	5.31 (1.24)	5.47 (1.32)	4.38 (1.13)
36 h after time zero	4.93 (0.09)	6.02 (0.14)	5.84 (0.14)	5.14 (0.28)
48 h after time zero	5.36 (1.03)	5.08 (1.44)	5.60 (1.53)	4.75 (0.50)
Chloride (mmol/l)				
Baseline	99.6 (3.3)	99.7 (2.8)	99.4 (3.2)	97.5 (2.5)
Nadir of the insult	101.0 (4.4)	100.7 (4.0)	99.8 (2.9)	97.4 (2.9)
12 h after time zero	98.3 (3.9)	96.9 (2.8)	96.5 (3.2)	97.3 (8.1)
24 h after time zero	97.7 (3.9)	94.7 (3.1)	97.4 (1.9)	95.0 (2.3)
36 h after time zero	100.1 (7.0)	95.8 (4.0)	98.0 (2.3)	100.0 (5.8)
48 h after time zero	100.0 (7.4)	96.3 (2.4)	99.0 (2.3)	101.0 (3.7)
Hematocrit				
Baseline	28.6 (10.6)	24.6 (3.3)	24.7 (4.7)	23.6 (6.0)
Nadir of the insult	22.8 (4.0)	29.6 (9.3)	29.6 (9.3)	28.4 (5.7)
12 h after time zero	23.8 (7.4)	22.3 (4.4)	27.7 (3.3)	29.3 (10.4)
24 h after time zero	22.6 (3.4)	27.6 (5.8)	22.6 (5.1)	31.7 (6.7)*,†
36 h after time zero	21.0 (3.8)	22.4 (3.2)	19.4 (5.0)	23.4 (5.5)
48 h after time zero	22.0 (4.5)	23.5 (7.3)	18.4 (5.3)	19.3 (5.6)
Hemoglobin (g/dl)				
Baseline	6.7 (3.6)	8.3 (1.1)	8.4 (1.6)	8.1 (2.0)
Nadir of the insult	7.8 (1.4)	7.8 (1.4)	10.0 (3.2)	9.6 (1.9)
12 h after time zero	8.1 (2.5)	7.6 (1.5)	9.4 (1.1)	11.1 (2.2)**
24 h after time zero	7.7 (1.2)	9.4 (2.0)	7.7 (1.7)	10.8 (2.3)*
36 h after time zero	7.1 (1.3)	7.6 (1.1)	6.6 (1.7)	8.0 (1.9)
48 h after time zero	7.5 (1.6)	8.0 (2.5)	6.7 (1.4)	6.6 (1.9)
Creatinine (mmol/l)				
Baseline	0.60 (0.13)	0.59 (0.07)	0.65 (0.24)	0.56 (0.07)
Nadir of the insult	0.58 (0.17)	0.53 (0.08)	0.64 (0.18)	0.52 (0.16)
12 h after time zero	0.73 (0.27)	0.90 (0.16)	0.95 (0.31)	0.80 (0.26)
24 h after time zero	0.94 (0.61)	1.20 (0.23)	0.99 (0.55)	1.2 (0.42)
36 h after time zero	1.17 (0.61)	1.38 (0.26)	1.50 (0.61)	1.24 (0.18)
48 h after time zero	1.53 (0.73)	1.54 (0.79)	1.57 (0.71)	1.00 (0.37)

Mean (SD) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and *post hoc* analysis were carried out on comparisons between groups with Tukey's and Dunett's method. Time zero was set at the start of resuscitation after the hypoxic–ischemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 minutes of resuscitation.

HI, hypoxia–ischemia.

*$P < 0.05$ vs. normothermia at the same time point or during the same time period. **$P < 0.05$ vs. 35 °C at the same time point or during the same time period. †$P < 0.05$ vs. 33.5 °C at the same time point or during the same time period.

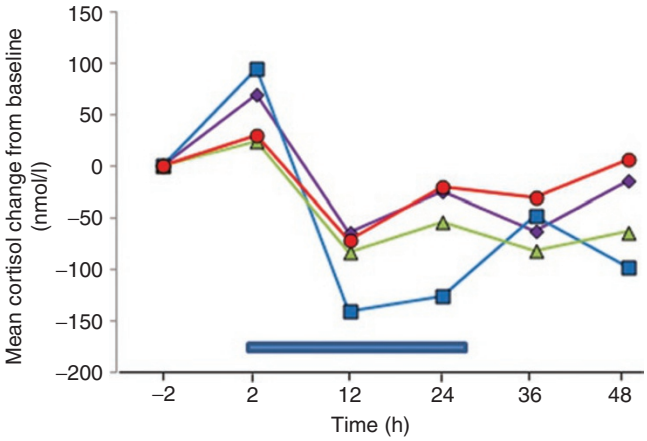

Figure 4. Mean serum cortisol change from baseline (nmol/l) at baseline, 2 h, and every 12 h post-HI for each temperature group: 38.5 °C (purple diamonds), 35 °C (blue squares), 33.5 °C (green triangles), and 30 °C (red circles). The blue bar represents the duration of the cooling period. HI, hypoxia–ischemia.

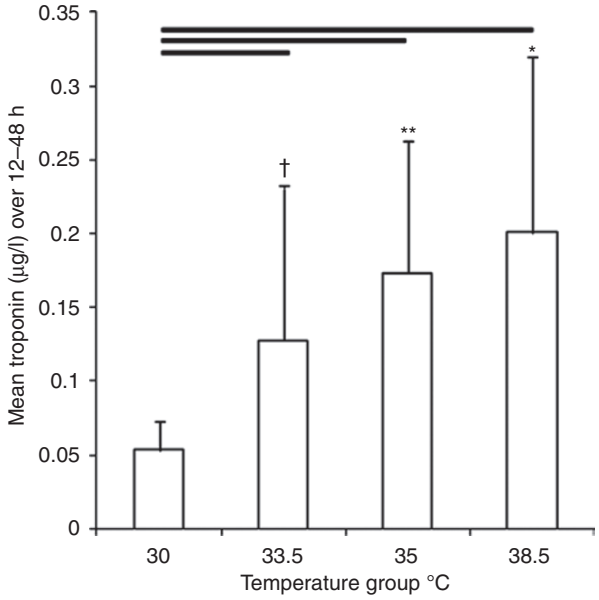

Figure 5. Mean serum troponin (µg/l) over 12–48 h. Serum cardiac troponin-I was significantly lower in the 30 °C group vs. the 38.5 °C; (*$P = 0.01$, 35 °C; **$P < 0.01$, and 33.5 °C; †$P = 0.01$) groups. Error bars represent ± 1 SD.

blood acid–base management. It is possible, however, that α-stat management would have reduced the adverse cardiac events because the protein charge with pH-stat under hypothermic conditions will resemble that seen in acidotic hearts during normothermia, which depresses cardiac function.

In summary, in our piglet model of perinatal asphyxia, we demonstrated abnormal metabolic homeostasis (lactic acidosis, hyperglycemia, and hypokalemia), increased need for inotrope and fluid bolus support to maintain MABP, and more fatalities with 30 °C cooling as compared with normothermia or cooling to 35 °C or 33.5 °C. In the clinical situation, induction and maintenance of mild therapeutic hypothermia are occasionally associated with unintentional overcooling, which may be damaging. The threshold safe therapeutic temperature may be influenced by insult severity and multiorgan dysfunction; however, our results suggest that prolonged cooling below 33.5 °C should be avoided.

METHODS

Animal Experiments and Surgical Preparation

All experimentation was under UK Home Office Guidelines (Animals (Scientific Procedures) Act 1986) and approved by the institutional animal care and use committee of the University College London Biological Services and Institute of Neurology. Twenty-eight large white male piglets aged <24 h (details in **Table 1**) were anesthetized and surgically prepared as described previously (39). Briefly, piglets were sedated with intramuscular midazolam (0.2 mg/kg), and arterial O_2 saturation was monitored (Nonin Medical, Plymouth, MN). Isoflurane anesthesia (4% vol/vol) was applied via a facemask during tracheostomy and intubation and maintained (3% during surgery, 2% otherwise). Piglets were mechanically ventilated so as to maintain the arterial pressures of O_2 (PaO_2, 8–13 kPa) and CO_2 ($PaCO_2$, 4.5–6.5 kPa), allowing for temperature correction of the arterial blood sample.

An umbilical venous catheter was inserted to infuse maintenance fluids (10% dextrose, 60 ml/kg/d), fentanyl (3–6 µg/kg/h), and antibiotics (benzylpenicillin 50 mg/kg and gentamicin 2.5 mg/kg, every 12 h). An umbilical arterial catheter was inserted for continuous HR and MABP monitoring and 6-h blood sampling to measure PaO_2, $PaCO_2$, pH, electrolytes, glucose (3–10 mmol/l), and lactate (Abbott Laboratories, Maidenhead, UK) (**Table 3** and **Table 4**). Bolus infusions of colloid (Gelofusin; B Braun Medical, Emmenbrucke, Switzerland) and inotropes (dopamine and dobutamine 5–20 µg/kg·min, noradrenaline 0.1–5 µg/kg·min) maintained MABP > 40 mm Hg. Hyperglycemia (>10 mmol/l) was treated by changing from 10% to 5% glucose; hyperglycemia (>20 mmol/l) was treated by using saline. Metabolic acidosis (base excess > −10) was corrected with

Table 5. Macroscopic organ pathology following hypoxia–ischemia and survival to 48 h according to temperature group

Group	Heart[a]	Lung	Liver	Kidney	Spleen and pancreas
38.5 °C, $n = 6$	N/A	1× pneumonia	No pathology seen	No pathology seen	No pathology seen
35 °C, $n = 7$	N/A	1× pneumonia	No pathology seen	Vacuolated	No pathology seen
33.5 °C, $n = 7$	No pathology seen	3× pneumonia	2× steatosis	Vacuolated; 2× acute tubular necrosis	No pathology seen
30 °C, $n = 7$	No pathology seen	No pathology seen	1× steatosis; 1× acute tubular necrosis	Vacuolated; 1× acute tubular necrosis	Severe patchy necrosis in pancreas
Naive, $n = 2$	No pathology seen	No pathology seen	No pathology seen	No pathology seen	No pathology seen

A subset of 27 piglets and two sets of naive piglet organs (lungs, liver, kidney, spleen, pancreas, and heart) were assessed for macroscopic pathology (×4 and ×40 magnification) and incidence of remarkable pathology per individual was noted.

N/A, not available.

[a]Only four hearts were available for analysis (one from the 33.5 °C group, one from the 30 °C group, two naive).

sodium bicarbonate (8.4% wt/vol). All animals received continuous physiological monitoring (SA instruments, Stony Brook, NY), and intensive life support throughout experimentation. Arterial lines were maintained by infusing 0.9% saline solution (Baxter, 1 ml/h) with heparin sodium (1 IU/ml) to prevent line blockage. Both common carotid arteries were surgically isolated at the level of the fourth cervical vertebra and encircled by remotely controlled vascular occluders (OC2A; In Vivo Metric, Healdsburg, CA). After surgery, piglets were positioned prone in a plastic pod with their heads immobilized.

Cerebral HI

A magnetic resonance spectroscopy surface coil was secured to the cranium, and the animal was positioned in a 9.4 Tesla Varian magnetic resonance spectrometer. While in the spectrometer, transient HI was induced by inflating the vascular occluders and reducing fractional inspired oxygen (F_iO_2) to 12% (vol/vol). During HI, cerebral-energetics were monitored every 2 min by phosphorus (^{31}P) magnetic resonance spectroscopy, and the β-nucleotide triphosphate (β-NTP; mainly adenosine triphosphate) peak height was automatically measured. When β-NTP had fallen to 40% of baseline, F_iO_2 was adjusted in order to stabilize β-NTP at that level for 12.5 min, after which the occluders were deflated and F_iO_2 normalized. ^{31}P spectra were acquired for a further 1 h to monitor recovery from HI.

The time integral of the decrement of β-NTP/EPP (EPP = exchangeable phosphate pool = inorganic phosphate + phosphocreatine + (2γ + β)-NTP) during HI and the first 1 h of resuscitation quantified the acute energy depletion as described previously (39).

Experimental Groups

Following HI and resuscitation piglets were randomized into four groups: (i) normothermia (rectal temperature (T_{rec}) 38.5 °C throughout), or whole-body cooling 2–26 h post-insult to (ii) T_{rec} 35 °C, (iii) T_{rec} 33.5 °C, or (iv) T_{rec} 30 °C (all groups $n = 7$). Normothermic piglets were maintained at their target T_{rec} using a warmed water mattress above and below the animal; hypothermia piglets were cooled (by reducing the water mattress temperature) to their target T_{rec} over 90 min starting 2 h after HI. At 26 h after HI, cooled piglets were rewarmed to normothermia at 0.5 °C/h using a water mattress with circulating water titrated to different temperatures with a heater. Forty-eight hours following HI, piglets were euthanized with pentobarbitol, the brain was cardiac perfusion–fixed with cold 4% paraformaldehyde and removed along with major organs and processed for histology and immunohistochemistry.

Serum Cortisol and Troponin

Arterial blood (1 ml) was taken every 12 h, allowed to clot for 60 min then spun at 4,000 rpm for 15 min. Serum supernatant was removed and separated into two eppendorf tubes and stored at −80 °C prior to analysis for cortisol and troponin levels.

Serum cortisol was determined using solid-phase, competitive chemiluminescent enzyme immunoassay (Immulite assay; Siemens, Camberley, UK). Three controls were used in each run (low, medium, and high). A sample was combined with polyclonal rabbit anticortisol antibody (Siemens) and pipetted into the test unit; alkaline phosphatase (bovine calf intestine) was conjugated to cortisol in buffer with preservative. After 30-min incubation, the test unit was cleaned and chemiluminescent substrate added. The light emitted was read after another 10 min.

Serum cardiac troponin 1 was determined using the chemiluminescent microparticle immunoassay with cross-reactivity with porcine troponin 1 (Abbott Architect STAT Troponin-I assay, sensitivity: ≤ 0.01 ng/ml, specificity: ≤ 1% with cardiac troponin-C and cardiac troponin-T, Abbott Laboratories). First, serum sample, assay diluent, and anti-troponin-I antibody–coated paramagnetic microparticles were combined. Second, after incubation and wash, anti-troponin-I acridinium-labeled conjugate was added. Following further incubation and wash, pre-trigger and trigger solutions were added to the reaction mixture. The resulting chemiluminescent reaction was measured as relative light units. A direct relationship existed between the amount of troponin-I in the sample and the relative light units detected by the Architect i* system optics. The concentration of troponin-I was read relative to a standard curve established with calibrators of known troponin-I concentrations.

Pathology

A subset of 27 animals and two naive controls (no surgery, no HI) had major organs (lung, liver, pancreas, spleen, kidney, and 2 × heart) assessed qualitatively for macropathology at low (×4) and high magnification (×40) by an expert pathologist (N. Sebire).

Data Analysis

Three periods were defined for HR and MABP analysis to obtain information about ideal cooling kinetics; cooling induction (2 to 3.5 h post-insult), cooling maintenance (3.5 to 26 h post-insult) and rewarming/normothermia (26 to 48 h post-insult). Blood chemistry analysis was performed at baseline, at the end of HI, and at 12, 24, and 48 h post-HI.

Intergroup statistical comparison of physiological measures and blood chemistry used linear regression and one-way ANOVA with adjustment for group baseline differences. Secondary "pairwise" analyses comparing the 30 °C group to each of the other groups included Bonferroni correction. Differences between the 30 °C group and the other groups are reported with a 98.3% CI. For non-normally distributed data, the Kruskal–Wallis rank test was used. Results are mean (±SD) unless stated otherwise; statistical significance was assumed for $P < 0.05$.

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