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Systemic effects of whole-body cooling to 35°C, 33.5°C and 30°C in a piglet perinatal asphyxia model: implications for therapeutic hypothermia

Aron Kerenyi[1], **Dorottya Kelen**[1], **Stuart D Faulkner**[1], **Alan Bainbridge**[2], **Manigandan Chandrasekaran**[1], **Ernest B Cady**[2], **Xavier Golay**[3], and **Nicola J Robertson**[1]

[1]Institute for Women's Health (AK, DK, SDF, MC, NJR), University College London, London WCIE 6AU, UK

[2]Medical Physics and Bio-engineering (AB, EBC), University College London, London WC1E 6DB, UK

[3]Institute of Neurology (XG), University College London, London WC1N 3BG, UK

Abstract

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 perinatal asphyxia model. Twenty-eight anesthetised male piglets aged <24h underwent hypoxia-ischemia and randomized to normothermia; or cooling to rectal temperature (Trec) 35°C, 33.5°C, or 30°C during 2-26 h post insult (groups n=7). Heart rate (HR), mean arterial blood pressure (MABP) and Trec were recorded continuously. Five 30°C animals had fatal cardiac arrests. During 30°C cooling HR was lower vs normothermia ($p<0.001$). Although MABP did not vary between groups, more fluid boluses were needed at 30°C than normothermia ($p<0.02$); dopamine use was higher at 30°C than normothermia and 35°C ($p=0.005$, $p=0.02$). Base deficit was increased at 30°C at 12,24 and 36h vs all other groups ($p<0.05$), pH was acidotic at 36h vs normothermia ($p=0.04$) and blood glucose higher for 30°C at 12h vs normothermia and 35°C ($p<0.05$). Potassium was lower at 12h in the 30°C group vs 33.5°C and 35°C groups. Cortisol was no different between groups. Cooling to 30°C led to metabolic derangement, more cardiac arrests and deaths than cooling to 33.5°C or 35°C. Inadvertent overcooling should be avoided.

Introduction

Therapeutic hypothermia is established as a safe and effective treatment for moderate to severe neonatal encephalopathy in the developed world (1); its clinical introduction follows decades of carefully conducted pre-clinical studies (2) and clinical trials (3-7). In these trials,

Corresponding author: Nicola J Robertson, Reader in Translational Neonatal Medicine and Honorary Consultant Neonatologist, Institute for Women's Health, University College London, 74 Huntley Street, London WC1E 6AU, United Kingdom, Tel: ++44 207 679 6052, Fax: ++44 207 380 7420, n.robertson@ucl.ac.uk.

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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-ischaemic insult. Both 32°C and 34°C reduce impairments (histological and behavioural) assessed 6 months after brief forebrain ischemia in the gerbil (2). In a small study of fetal sheep, with cooling starting 90 min after hypoxia-ischemia (HI), protection was only seen with a sustained extradural temperature ≤34°C (8) and in the piglet, 35°C provided better neuroprotection in deep grey matter than 33°C (9). However, in adult primates 48h at 29°C following middle-cerebral artery occlusion was associated with worse outcomes (100% fatality) compared to 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 where studies were grouped into those with a target core temperature >34°C and ≤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 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 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 centres. 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 centre (15); other pilot studies have intentionally targeted core temperatures of 32.2°C (+/− 0.9) for 72h (16).

The aim of the present study was to assess the systemic effects of cooling for 24h 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).

Materials and Methods

Animal experiments and surgical preparation

All experimentation was under UK Home Office Guidelines (Animals (Scientific Procedures) Act 1986) and Institutional Animal Care and Use Committee (University College London Biological Services and Institute of Neurology). Twenty-eight large-white male piglets aged <24h (details in Table 1) were anesthetised and surgically prepared as described previously (17). Briefly, piglets were sedated with intramuscular midazolam (0.2mg/kg) and arterial O_2 saturation was monitored (Nonin Medical,USA). Isoflurane

anesthesia (4% volume/volume (v/v)) was via a facemask to facilitate tracheostomy and intubation and was 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, 60ml/kg/day), fentanyl (3-6 μg/kg/h), and antibiotics (benzylpenicillin 50mg/kg and gentamicin 2.5mg/kg, every 12h). An umbilical arterial catheter was inserted for continuous heart rate (HR) and mean arterial blood pressure (MABP) monitoring and 6hrly blood sampling to measure PaO_2, $PaCO_2$, pH, electrolytes, glucose (3-10 mmol/l), and lactate (Abbot Laboratories, UK) (Table 2 and Table 3). Bolus infusions of colloid (Gelofusin, B Braun Medical Ltd. Emmenbrucke, Switzerland) and inotropes (dopamine and dobutamine 5-20ug/kg.min, noradrenaline 0.1-5 ug/kg.min) maintained MABP >40mmHg. Hyperglycemia (>10mmol/l) was treated by changing from 10% to 5% glucose; hyperglycemia (>20mmol/l) was treated by using saline. Metabolic acidosis (Base Excess (BE) > −10) was corrected with sodium bicarbonate (8.4% weight/volume (w/v)). All animals received continuous physiological monitoring (SA instruments, New York, USA), and intensive life support throughout experimentation. Arterial lines were maintained by infusing 0.9% saline solution (Baxter, 1ml/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, California, USA). After surgery, piglets were positioned prone in a plastic pod with their heads immobilised.

Cerebral hypoxia-ischemia

A magnetic resonance spectroscopy (MRS) surface coil was secured on the cranium and the animal was positioned in a 9.4 Tesla Varian magnetic resonance spectrometer. Whilst in the spectrometer, transient hypoxia-ischaemia was induced by inflating the vascular occluders, and reducing fractional inspired oxygen ($F_i O_2$) to 12% (v/v). During hypoxia-ischaemia cerebral-energetics were monitored every 2 min by phosphorus (^{31}P) MRS and the beta-nucleotide triphosphate (β-NTP; mainly ATP) peak height was automatically measured. When β-NTP had fallen to 40% of baseline F_iO_2 was adjusted in order to stabilise β-NTP at that level for 12.5 min after which the occluders were deflated and F_iO_2 normalised. ^{31}P spectra were acquired for a further 1h to monitor recovery from hypoxia-ischaemia.

The time integral of the decrement of β-NTP/EPP (EPP = exchangeable phosphate pool = inorganic phosphate + phosphocreatine (PCr) + (2γ + β)-NTP) during HI and the first 1h of resuscitation quantified the acute energy depletion (AED) as described previously (17).

Experimental groups

Following hypoxia-ischaemia and resuscitation piglets were randomised into 4 groups: i) normothermia (rectal temperature (T_{rec}) 38.5°C throughout), or whole-body cooling 2-26h 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 2h after HI. At 26h after hypoxia ischaemia, cooled piglets were re-warmed 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 hypoxia-ischaemia, piglets were euthanized with pentobarbitol, the brain was cardiac perfuse fixed with cold 4% paraformaldehyde and removed along with major organs and processed for histology and immunohistochemistry.

Serum cortisol and troponin

Arterial blood (1ml) was taken every 12h, allowed to clot for 60min then spun at 4000rpm for 15mins. Serum supernant was removed and separated into two eppindorffs 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 machine). Three controls were used in each run (low, medium and high). A sample was combined with polyclonal rabbit anticortisol antibody (Seimens) and pipetted in the test unit; alkaline phosphatase (bovine calf intestine) conjugated to cortisol in buffer, with preservative. After 30 minutes incubation the test unit was cleaned and chemiluminescent substrate added. The light emitted was read after another 10 minutes.

Serum cardiac troponin 1 (cTn1) was determined using the chemiluminescent microparticle immunoassay (CMIA) with cross reactivity with porcine Tn1 (Abbott ARCHITECT STAT Troponin-I assay, sensitivity; ≤ 0.01 ng/ml, specificity: ≤ 1% with cardiac troponin-C and cardiac troponin-T, Abbott Laboratories, IL, USA). Firstly, serum sample, assay diluent and anti-troponin-I antibody-coated paramagnetic microparticles were combined. Secondly, 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 (RLUs). A direct relationship existed between the amount of troponin-I in the sample and the RLUs detected by the ARCHITECT i* system optics. The concentration of troponin-I is read relative to a standard curve established with calibrators of known troponin-I concentrations.

Pathology

A subset of 27 animals and 2 naïve 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 (NS).

Data analysis

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

Intergroup statistical comparison of physiological measures and blood chemistry used linear regression and one-way analysis of variance (ANOVA) with adjustment for group baseline

differences. Secondary ‘pairwise’ analyses comparing the 30°C group to each other group included Bonferroni correction. Differences between the 30°C group and the other groups are reported with a 98.3% confidence interval (CI). For non-normally distributed data Kruskal-Wallis rank test was used. Results are mean (SD) unless stated otherwise; statistical significance was assumed for $p<0.05$.

Results

There were no intergroup differences in body weight, postnatal age, insult severity or baseline physiological and biochemical measures (Table 1). Six 30°C piglets suffered an episode of cardiac arrest and five of these died before 48h post HI due to a combination of pulmonary edema, fluid in the thorax or abdominal cavities, severe metabolic derangement or profound hypotension. One 33.5°C animal suffered 2 cardiac arrests but survived to 48h. There was one recorded incidence of persistent arrhythmia in 30°C group.

Physiological measures

During cooling induction and re-warming/normothermia mean HR and MABP were similar in all groups (Table 1). Baseline HR averaged over all groups was 154 (27) 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 ($p<0.001$, Fig. 1a).

Several 30°C animals’ experienced periods of profound hypotension (~30mmHg) lasting several hours; these episodes were usually preceding cardiac arrest and were mainly experienced by the 5 animals in the 30 °C group that died before 48h. However, when averaged over each analysis period, the MABP was similar to baseline in all of the cooling groups (Fig. 1b).

Volume replacement was required in 2 normothermic animals following hypoxia-ischaemia: these animals required no inotropes. The overall median volume replacement (saline and Gelofusin) was higher for 30°C compared to normothermia ($p=0.05$; Kruskal Wallis and Mann Whitney) (Table 4). The median dopamine infusion dose over 48h was higher for 30°C cooling than for normothermia ($p=0.01$) or 35°C ($p=0.05$, Kruskal Wallis and Mann Whitney) (Table 4). The median dopamine infusion was also greater for 33.5°C cooling than for normothermia ($p=0.05$). In addition, multiple inotrope infusions (dopamine, dobutamine and adrenaline) were required during maintenance and re-warming/normothermia in the 30°C group while only dopamine was required in other groups.

Blood biochemistry

Base deficit was increased in the 30°C group at 12, 24 and 36h compared to other groups (all $p<0.05$; Table 2 and Fig 2a): base deficit was similar amongst all other groups at these times. Blood pH was lower in the 30°C group at 36h ($p=0.05$), with borderline post-hoc significance compared with the normothermic group at 12h ($p=0.05$, Table 2 and Fig 2b).

In the 30°C group blood glucose was higher at 12h than both the normothermic and 35°C groups ($p=0.05$ and $p=0.05$ respectively) but had normalised by 24h (Table 2 and Fig 3a and

b). Blood lactate was highest at 24h with 30°C cooling but was not significantly different from the other groups (Table 2 and Fig 3c and d). Blood potassium was lower in the 30°C group at 12h compared to 35 °C and 33.5°C (p= 0.01 and p=0.05 respectively). Haematocrit was greater in the 30°C group at 24h compared to normothermia and 33.5°C (both p=0.05) and haemoglobin greater in 30°C group at 12h compared to 35°C (p=0.05) and at 24h compared to 33.5°C (p=0.05) (Table 3).

Shivering episodes (non-seizure 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 2h following hypoxia-ischaemia and reduced at 12h (during the cooling phase) after hypoxia-ischaemia (Fig 4).

Mean cardiac troponin I levels were significantly lower in the 30°C group compared to all other groups over 12-48h following hypoxia-ischaemia ($p \leq 0.01$, Fig 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). Common findings across all groups were patchy sinusoidal congestion of the liver. 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 (ATN) and liver steatosis were limited to 33.5°C and 30°C groups only. No evidence of macroscopic pathology was noted in 2 hearts (30°C and 33.5°C). No organ pathology was noted in 2 naïve piglets.

Discussion

In our perinatal asphyxia piglet model we demonstrated abnormal metabolic homeostasis (lactic acidosis, hyperglycaemia, hypokalaemia), increased need for inotrope and fluid bolus support to maintain MABP, and more fatalities with 30°C cooling compared to 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 over-cooling 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, 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 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 carbon dioxide production (22); other mechanisms of hypothermic neuroprotection include: reduced excitotoxicity, calcium antagonism, protein synthesis preservation, decreased oedema, modulation of the inflammatory cascade and a change in pro and anti-apoptotic signalling (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 due to cardiac arrest and fatal arrhythmias, similar to those 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. Cooling to 33.5°C required more dopamine than normothermia and 35°C groups whereas cooling to 35°C had no increased requirements in volume or inotropes compared to normothermia. 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 non-cooled infants (26).

Pilot clinical studies and clinical trials of cooling to a core temperature of 33-34 °C have shown no physiological and clinical differences with mild cooling apart from higher incidences of arrhythmias and thrombocytopenia (12). Azzopardi et al (27) observed mild hypokalaemia in a pilot cooling study; hypothermia induced hypokalaemia has been seen commonly in experimental and clinical studies (28) and relates to the intracellular shift in potassium, increased sympathetic tone and stimulation of B2-adrenergic receptors (29). In piglets cooled to 30°C we observed hypokalemia at 12h. Increased hematocrit was seen in the 30°C group; this was unexpected in light of the large volume of fluid administered to maintain the 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 haemoglobin-oxygen dissociation curve would further enhance peripheral vasoconstriction, lactic acidemia and hypovolemia. Hyperglycemia produces enhanced cerebral injury in adult human and experimental models of hypoxia-ischaemia (30), although in neonatal models the influence of hyperglycemia on brain injury is more varied with several studies demonstrating a beneficial effect of glucose administration prior to or immediately after cerebral ischemia (31) with PCr and ATP preservation (32). We did not use muscle relaxants in this model and sedation and anaesthesia were administered at all times. A previous study demonstrated that cooling in un-sedated piglets results in increased cortisol levels, shivering and loss of hypothermic neuroprotection (33). 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 12-48h compared to 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 (34). Our data concur with the recent findings in another piglet asphyxia model where cooling to 34.5°C after the injury reduced serum cardiac troponin levels by 6h compared to normothermia (35). 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 pre-existing severe multi-organ damage. The threshold minimum safe cooling temperature may therefore depend on brain injury severity and hypoxic-ischaemic organ dysfunction.

In clinical practice, it is important to differentiate *accidental* from *therapeutic* hypothermia. The World Health Organisation reported global neonatal mortality rates of 39%, 52% and 80% respectively with mild, moderate and severe hypothermia at hospital presentation (36). 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 centres 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) show that therapeutic hypothermia reduces death or disability at 18 months with a risk ratio of 0.81 (95% 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 72h in a pilot study (16). A large trial is in progress evaluating deeper and longer cooling periods (32°C for 120h vs 33.5°C for 72h) for infants presenting aged < 6h (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, compared to 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 ~2h after HI and maintained it for 24h. Our hypothermia initiation delay is shorter than the median time of recruitment for the randomised 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 centre. In addition, our cooling duration is shorter than current cooling protocols (72h), however 24h cooling offers comparable neuroprotective efficacy in the piglet and is practical and feasible. Following hypoxia-ischaemia in the piglet, we do not generally observe a lactic acidosis in the piglet model; this differs to 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 localised ischaemic and global hypoxic insult in the piglet asphyxia model.

The anesthesia used in our model (isoflurane 1–4%) constitutes a difference between experimentation and clinical protocols. Gaseous anesthetics decrease cardiac output, stroke

volume, and ejection fraction by ~75% from conscious levels (37). We cannot exclude an interaction between different degrees of hypothermia and isoflurane which 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 pre-clinical model. Finally, we used pH stat for acid base management because in pre-clinical piglet (38) and clinical studies (39) neurologic recovery was improved with pH stat compared to alpha stat blood acid base management. It is possible, however, that alpha-stat management would have reduced the adverse cardiac events since 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 perinatal asphyxia piglet model we demonstrated abnormal metabolic homeostasis (lactic acidosis, hyperglycaemia, hypokalaemia), increased need for inotrope and fluid bolus support to maintain MABP, and more fatalities with 30°C cooling compared to 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 over cooling which may be damaging. The threshold safe therapeutic temperature may be influenced by insult severity and multi-organ dysfunction; however, our results suggest that prolonged cooling below 33.5°C should be avoided.

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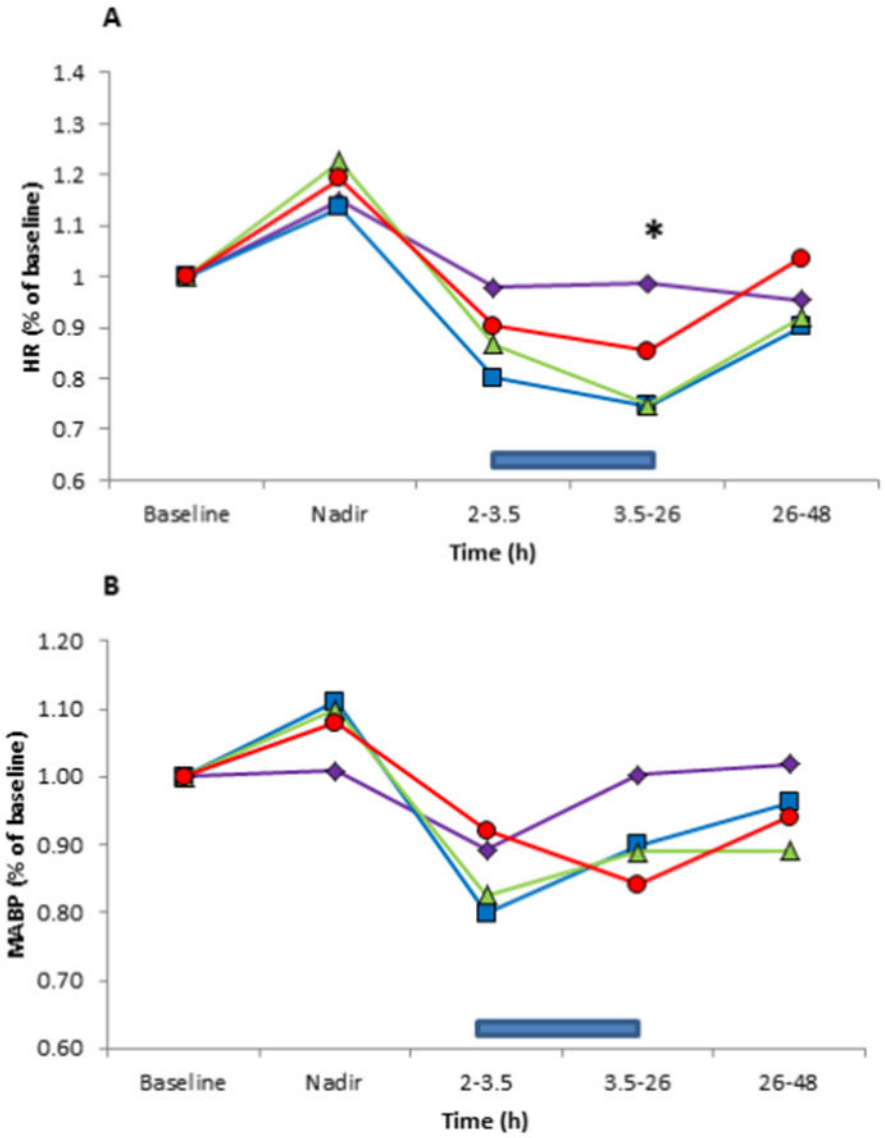

Figure 1. Mean % change in (a) heart rate and (b) mean arterial blood pressure during and after 24h whole body hypothermia for each temperature group; 38.5°C (◆, purple), 35°C (■, blue) 33.5°C (▲, green) and 30°C (•, red) following transient hypoxia ischemia.

A blue bar represents the duration of the cooling period. * significant difference between 30°C and normothermia (38.5°C) ($p<0.001$). Nadir; hypoxia-ischemia midpoint. Time 0h; end of hypoxia-ischemia

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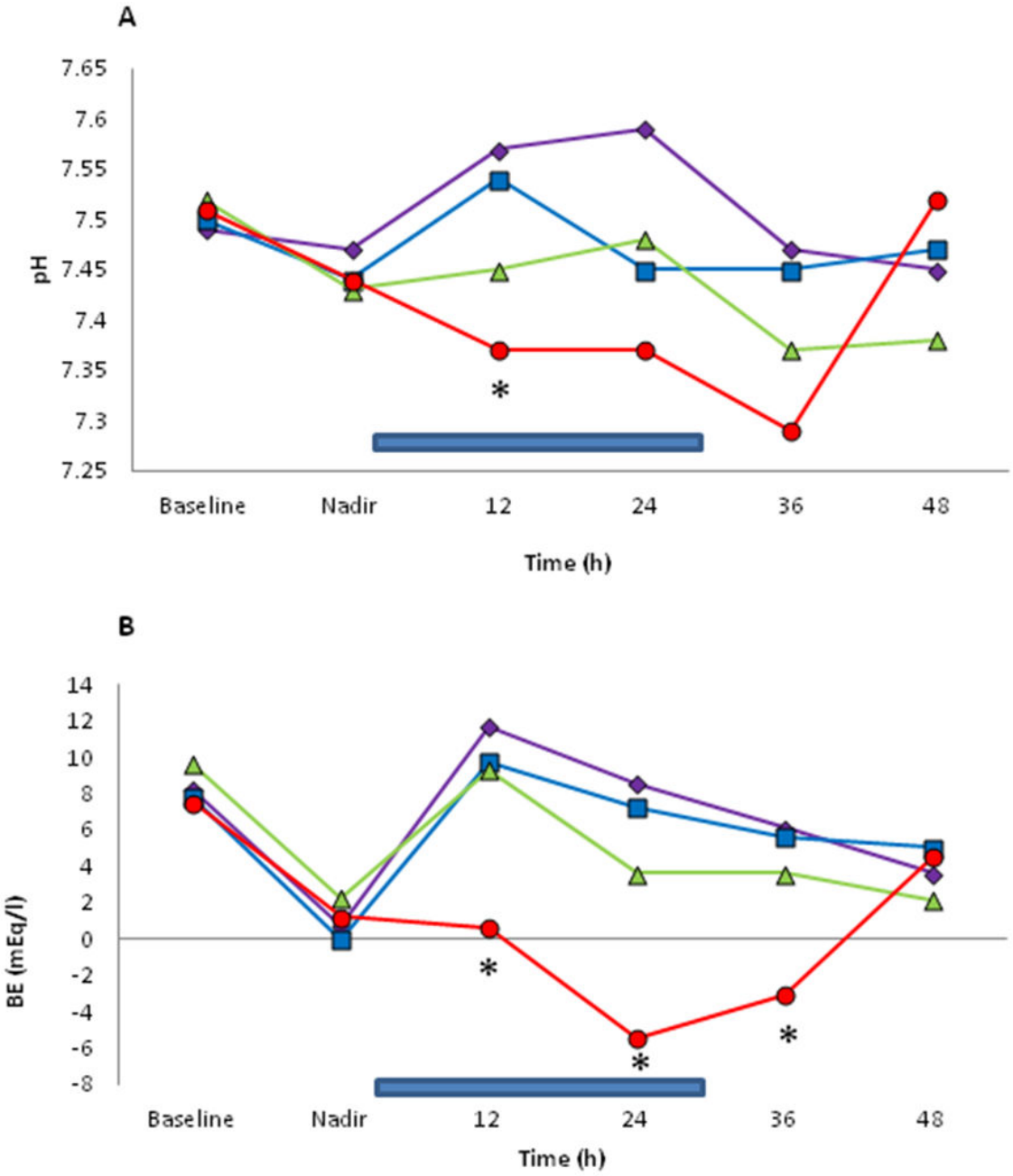

Figure 2. Mean (a) base excess (BE) and (b) blood pH at baseline, nadir and 12, 24 and 48h post HI for each temperature group; 38.5°C (◆, purple), 35°C (■, blue) 33.5°C (▲, green) and 30°C (•, red).

A blue bar represents the duration of the cooling period. * significant difference between 30°C and all other groups ($p<0.05$) at 12, 24 and 36h Nadir; hypoxia-ischaemia midpoint. Time 0h; end of hypoxia-ischemia

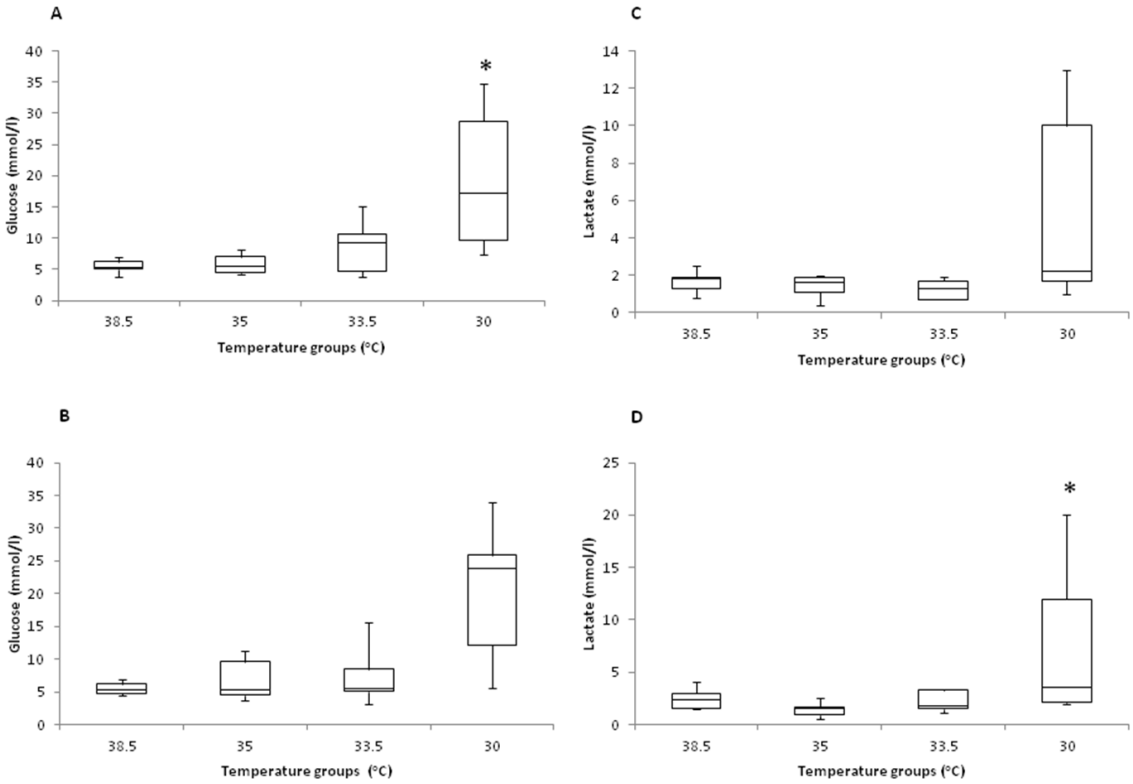

Figure 3. Median blood glucose (a and b) and lactate (c and d) levels at 12 and 24h post HI.
12h * significant difference between 30°C group vs normothermia and 35°C group for glucose only ($p<0.05$).

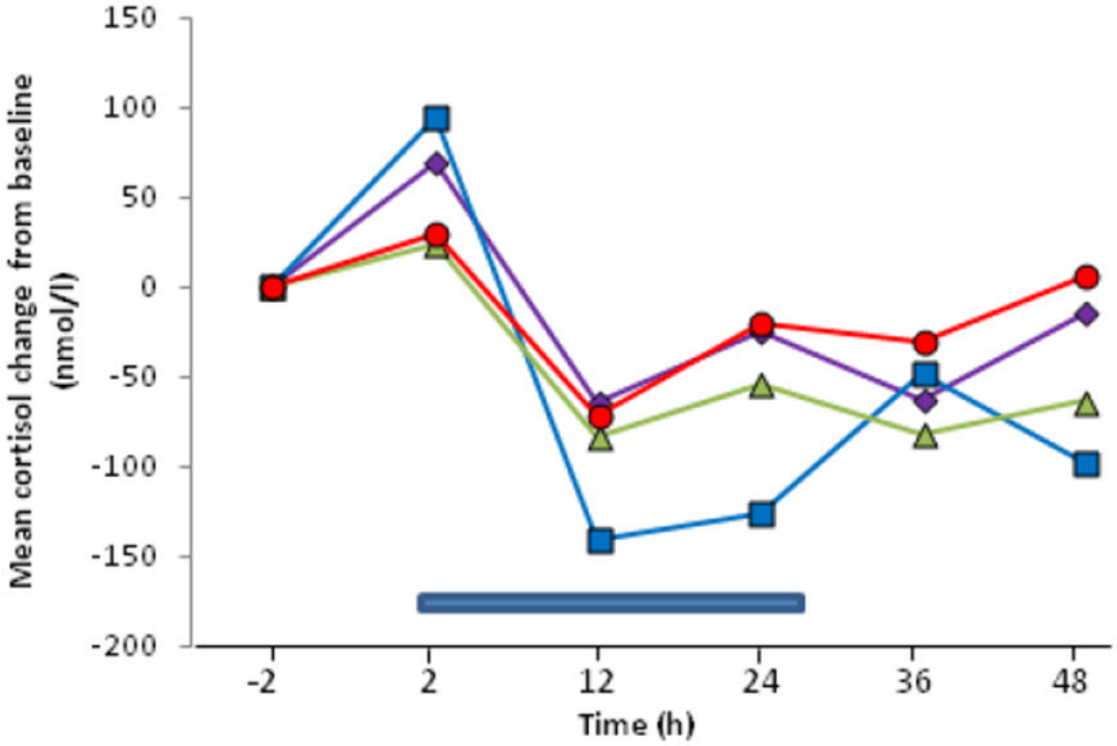

Figure 4. Mean serum cortisol change from baseline (nmol/l) at baseline, 2h and every 12h post HI for each temperature group; 38.5°C (◆, purple), 35°C (■, blue) 33.5°C (▲, green) and 30°C (•, red).

A blue bar represents the duration of the cooling period.

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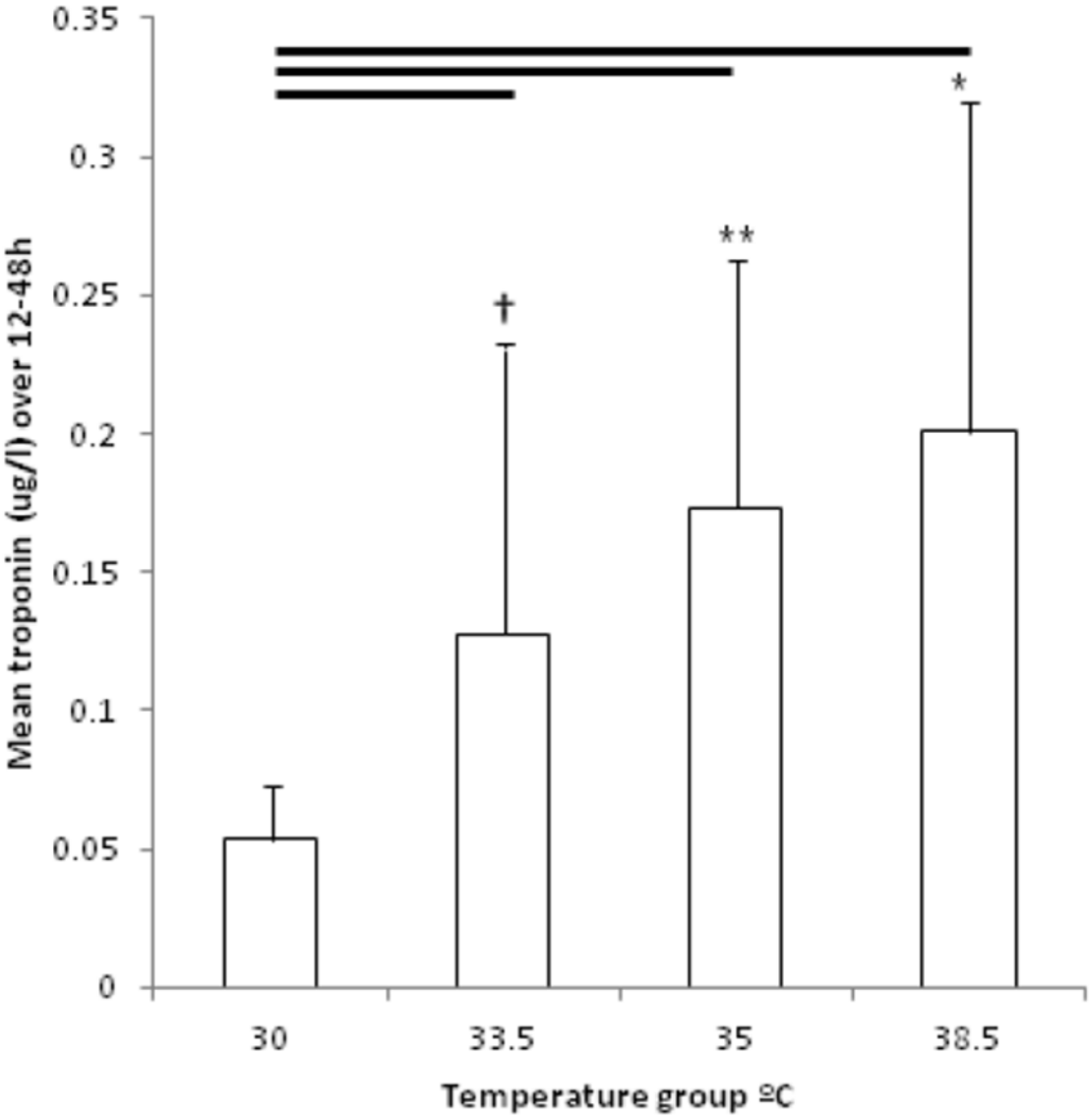

Figure 5. Mean serum troponin (ug/l) over 12-48h

Serum cardiac Troponin I was significantly lower in 30°C group vs * 38.5°C; (p=0.01, ** 35°C; P<0.01, and † 33.5°C; p=0.01). Error bars represent +/− 1 standard deviation (SD).

Table 1
Physiological variables for piglets in each temperature group

Variables	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	1771 (132)	1786 (90)	1714 (146)	1864 (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 (mmHg)				
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 was 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-ischaemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 minutes of resuscitation.

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

Table 2
Blood gas variables for piglets in each temperature group

Variables	Normothermic	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 Excess (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)

Variables	Normothermic	35°C	33.5°C	30°C
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) and median (interquartile range (IQR)) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and post hoc analysis was 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-ischaemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 minutes of resuscitation.

PaO_2 = partial pressure arterial of oxygen; $PaCO_2$ =partial arterial pressure of carbon dioxide.

* $p<0.05$ 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

§ $p<0.05$ within group comparisons vs baseline

§§ $p<0.05$ within group comparisons vs 24h after time zero

Table 3
Blood chemistry for piglets in each temperature group

Variables	Normothermic	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)				

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Variables	Normothermic	35°C	33.5°C	30°C
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) and median (interquartile range (IQR)) values are presented for each group. Linear regression with adjustments to baseline and one-way ANOVA and post hoc analysis was 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-ischaemic insult. The insult severity index was defined as the time integral of the acute energy depletion during HI and for 60 minutes of resuscitation.

* $p<0.05$ 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.

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Table 4

Median (IQR) total volume replacement (ml/kg) and inotrope dose (ug/kg/min) over the 48 hour period following hypoxia-ischaemia according to each temperature group

	Normothermic	35 °C	33.5 °C	30 °C
Volume replacement (ml/kg)	15 (0, 31)	19 (0, 38)	18 (0, 82)	70 (46-108) *
Inotropes (ug.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.

* $p<0.05$ 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.

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Table 5

Macroscopic organ pathology following hypoxia-ischemia and survival to 48 hours according to temperature group

Group	Heart *	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
Naïve **n=2**	no pathology seen	no pathology seen	no pathology seen	no pathology seen	no pathology seen

A subset of 27 piglets and 2 naïve 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 were noted.

* Only 4 hearts were available for analysis (33.5°C, 30°C, 2 naïve).