Background and Purpose—In infants with moderate to severe neonatal encephalopathy, whole-body cooling at 33°C to 34°C for 72 hours is standard care with a number needed to treat to prevent adverse outcomes of 6 to 7. The precise brain temperature providing optimal neuroprotection is unknown.

Methods—After a quantified global cerebral hypoxic-ischemic insult, 28 piglets aged <24 hours were randomized (each group, n=7) to (1) normothermia (38.5°C throughout) or whole-body cooling 2 to 26 hours after insult to (2) 35°C, (3) 33.5°C, or (4) 30°C. At 48 hours after hypoxia-ischemia, delayed cell death (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling and cleaved caspase 3) and microglial activation (ionized calcium-binding adapter molecule 1) were evaluated.

Results—At 48 hours after hypoxia-ischemia, substantial cerebral injury was found in the normothermia and 30°C hypothermia groups. However, with 35°C and 33.5°C cooling, a clear reduction in delayed cell death and microglial activation was observed in most brain regions (P<0.05), with no differences between 35°C and 33.5°C cooling groups. A protective pattern was observed, with U-shaped temperature dependence in delayed cell death in periventricular white matter, caudate nucleus, putamen, hippocampus, and thalamus. A microglial activation pattern was also seen, with inverted U-shaped temperature dependence in periventricular white matter, caudate nucleus, internal capsule, and hippocampus (all P<0.05).

Conclusions—Cooling to 35°C (an absolute drop of 3.5°C as in therapeutic hypothermia protocols) or to 33.5°C provided protection in most brain regions after a cerebral hypoxic-ischemic insult in the newborn piglet. Although the relatively wide therapeutic range of a 3.5°C to 5°C drop in temperature reassured, overcooling (an 8.5°C drop) was clearly detrimental in some brain regions. (Stroke. 2015;46:275-278. DOI: 10.1161/STROKEAHA.114.007330.)

Key Words: hypothermia ■ hypoxia-ischemia, brain ■ neonatal encephalopathy ■ neuroprotection

Therapeutic hypothermia is now standard clinical care for moderate to severe neonatal encephalopathy in the United Kingdom and developed world.1 Clinical trials have included whole-body cooling with core temperature reduced to 33.5°C for 72 hours2 because the optimal temperature for neural rescue is likely to be below 34°C. There are, however, 40–50% of infants who, despite hypothermic treatment, have an adverse neurodevelopmental outcome.1 Cooling to lower temperatures has been suggested, particularly for those with severe encephalopathy,1 but the ideal temperature for brain protection is unknown.

The aim of the current study was to assess brain regional cell death and microglial activation under normothermia, and with cooling to 35°C, 33.5°C, and 30°C from 2 to 26 hours after a global cerebral hypoxic-ischemic insult in the piglet asphyxia model.

Methods

Expanded Methods are available in the online-only Data Supplement. All experimentation was under UK Home Office Guidelines (Animals [Scientific Procedures] Act 1986) and approved by the Animal Care and Use Committee of University College London Biological Services and Institute of Neurology.

The hypoxic-ischemia insult was performed in 28 large white male piglets aged <24 hours as previously described.4 After hypoxia-ischemia (HI) and resuscitation, piglets were randomized into 4 groups: (1) normothermia (rectal temperature [Trec], 38.5°C throughout) or whole-body cooling 2 to 26 hours after insult to Trec (2) 35°C, (3) 33.5°C; or (4) 30°C.
Normothermic piglets were maintained at their target Trec using a warmed water mattress above and below the animal; hypothermia piglets were cooled (by reducing the water mattress temperature) to their target Trec over 90 minutes starting 2 hours after HI. At 26 hours after HI, cooled piglets were rewarmed to normothermia at 0.5°C/h using a water mattress with circulating water heated to increasing temperatures. At 48 hours after HI, piglets were euthanized and 11 brain regions (Figure 1) were analyzed for histology and immunohistochemistry (online-only Data Supplement).

**Results**

There were no differences in body weight, postnatal age, insult severity, or baseline physiological and biochemical measures (data not shown) between the temperature groups. The physiological and systemic effects of cooling to different temperatures have been previously described in detail4 (Table I in the online-only Data Supplement).

As shown in Figure 2, there was a reduction in the number of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling positive cells in the 35°C and 33.5°C hypothermia groups when compared with the normothermic group, an effect being absent at 30°C. Figure 3 (extended in Table II in the online-only Data Supplement) shows that cooling at 33.5°C and 35°C resulted in lower terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling counts (compared with normothermia) in midtemporal cortex, periventricular white matter, caudate, putamen, hippocampus, and thalamus, with no differences between both cooling groups. Cooling at 30°C reduced cell death only in the hippocampus.

Cooling at 33.5°C reduced cleaved caspase 3–positive cells in hippocampus and thalamus in comparison with normothermia (Figure 3; extended in Table II in the online-only Data Supplement). Cooling at 30°C was not associated with lower cleaved caspase 3 counts. In the periventricular white matter, the numbers of cleaved caspase 3–positive cells were reduced in all temperature groups.

HI after normothermia resulted in the loss of the microglial branches, with many cells transforming into completely rounded brain macrophages (Figure 2, bottom). When compared with normothermia, cooling at 35°C or 33.5°C significantly preserved ramification index, showing a similar increase in ramification with no difference between groups. The 30°C group showed a lower ramification index than the 35°C and 33.5°C groups in most regions.

**Discussion**

This study demonstrated substantial brain injury after HI in newborn piglets subsequently maintained normothermic and also in those treated with 30°C hypothermia, but a clear reduction in cell damage by cooling to 35°C and 33.5°C. Moreover, a temperature-dependent protective pattern was observed in some brain regions, with U-shaped temperature dependence for delayed cell death and inverted U-shaped temperature dependence for microglial ramification.

![Figure 1. Piglet brain regions assessed for immunohistochemistry.](image)

![Figure 2. Representative photomicrographs from each temperature group at 48h after hypoxia-ischemia. Top, TUNEL-positive cells (thalamus). Middle, Cleaved caspase-3-positive cells (thalamus). Bottom, Ionized calcium-binding adapter molecule 1 (Iba-1) immunostaining (caudate).](image)
Apart from the commonly known decrease in the metabolic rate (7%–9% per 1°C core temperature reduction) with parallel decreases in O$_2$ consumption and CO$_2$ production, the beneficial effects of hypothermia include reduced excitotoxicity, calcium antagonism, protein synthesis preservation, decreased edema, modulation of the inflammatory cascade, and a change in proapoptotic and antiapoptotic signaling. However, hypothermia may also have adverse effects, such as reduced cardiac contractility, reduced cerebral blood flow, poor perfusion, sympathetic and neuroendocrine stimulation, and increased blood viscosity. It is likely, therefore, that an effective temperature range exists below which hypothermic neuroprotection is lost; this therapeutic temperature range may be influenced by severity, the delay in onset and duration of cooling, and other factors, such as the peripheral immune response.

Cooling from 38.5°C to 30°C (an 8.5°C absolute temperature drop) neither reduced delayed cell death nor maintained the microglial ramification index in most of the brain regions evaluated, showing a global neuropathological pattern similar to that for normothermia. These histological results accord
with physiological observations previously documented by our group, describing abnormal metabolic homeostasis with lactic acidosis, hyperglycemia, hypokalemia, and an increased need for inotrope and fluid bolus support to maintain the mean arterial blood pressure for 30°C cooling compared with other temperatures. Cooling from 38.5°C to 35°C or 33.5°C, i.e. a reduction of 3.5°C to 5°C, was associated with a similar profile of protection based on terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling staining, with no difference in the number of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling positive cells between the 35°C and 33.5°C cooling groups. For caspase positive cells, although a significant difference was seen for more regions after cooling to 33.5°C (hippocampus and thalamus), this must be taken in the context that caspase expression was seen in a subset of dead cells only and, therefore, the apparent difference may represent a change in the speed of evolution of death, not total death. These data may suggest that the extra 1.5°C drop in temperature is unlikely to be harmful, thus helping to explain why therapeutic hypothermia has been successful, despite considerable variation in the stringency of temperature control between clinical trials. However, some caution is needed because the Optimizing Cooling trial, which compared cooling deeper (32°C) and longer (120 hours) with conventional cooling protocols, was recently closed for safety and futility after 364 of planned 726 infants were enrolled after recommendation from the data and safety monitoring committee (http://clinicaltrials.gov/ct2/show/results/NCT01192776). The in-hospital mortality rate increased from 7% to 14%, when 72-hour cooling at 33.5°C and 32°C was compared for safety and futility after 364 of planned 726 infants were enrolled after recommendation from the data and safety monitoring committee (http://clinicaltrials.gov/ct2/show/results/NCT01192776).

Although the extent of cell death and tissue damage varies according to brain region, the overall neuroprotective effect of hypothermia is determined by the local tissue susceptibility to injury. In this study, cooling to 30°C led to worse injury in some brain areas, such as thalamus, putamen, and caudate nucleus; these data show a U-shaped temperature dependence of reduced delayed cell death and an inverted U-shaped temperature dependence of maintained microglial ramification. Previously described harmful side effects of hypothermia combined with the intrinsic cerebral metabolic features of the deep gray matter may render deep gray matter more vulnerable to injury from both HI and overzealous cooling. These peripheral effects are likely to occur to a different extent in neonatal encephalopathy and will depend on the extent of multiorgan failure and severity of HI.

It must be taken into consideration that the relative decrease in temperature in the present study was greater in our piglets (normal core temperature, 38.5°C) than would be the case for the same target core temperature in the human infant; relatively large temperature reduction and metabolic rate reduction were, therefore, induced in our piglet studies compared with encephalopathic human newborns cooled to 33.5°C. There are other important differences between species; for example, piglets have a higher basal cerebral metabolic rate of oxygen and greater metabolic rate reduction with hypothermia than human newborns. Whether it is better to induce the actual cooling temperature used in existing clinical protocols in human infants or induce the clinical temperature drop from the normothermic piglet temperature for these translational studies is unclear. Our cooling protocol (24 hours of hypothermia duration) is shorter than the current clinical protocol (72 hours of cooling), and further cell death may occur beyond the 48-hour time-point when we quantified it; nevertheless, several long-term studies have previously demonstrated a permanent protective effect.

This piglet asphyxia study of cooling from 2 to 26 hours after a global hypoxic-ischemic insult demonstrates that optimal neuronal and white matter protection was seen with a reduction in the core temperature of 3.5°C to 5°C (35°C and 33.5°C, respectively). Although the relatively wide therapeutic range of 1.5°C is reassuring, these data emphasize the potential detrimental effects of excessive cooling.

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References
Brain Cell Death Is Reduced With Cooling by 3.5°C to 5°C but Increased With Cooling by 8.5°C in a Piglet Asphyxia Model

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