

Profound Hypothermia Provides Neuroprotection Following Hypothermic Circulatory Arrest: Ultrastructural Observations

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ABSTRACT

Objective: To assess whether cooling to 10°C can reduce neurological injury during 75 minutes of hypothermic circulatory arrest (HCA) compared to cooling to 18°C.

Methods: Twelve domestic swine were used for this prospective blind randomized study. The animals were divided into 2 groups: Group A (n=6) underwent hypothermic circulatory arrest at 18°C for 75 min, and Group B (n=6) underwent hypothermic circulatory arrest at 10°C for 75 min. At the end of the experiment, the brains were removed and immersed in paraformaldehyde. All brains were dissected in the sagittal plane. Tissue blocks from the left hemisphere were cut to encompass the sensory neocortex.

Results: The selected area was identified with a dissecting microscope. Samples were examined in a blind fashion using electron microscope. Two investigators were instructed to find 10 representative neurons and analyze electron micrographs of these neurons for evidence of nuclear and cytoplasmic changes. Similarly, each investigator was instructed to examine the perinuclear neuronal mitochondria for abnormalities in mitochondrial distribution. Significant differences were observed between the 2 groups in mitochondria and rough endoplasmic reticulum (RER). In 5 of the 6 animals treated with 18°C HCA, neurons had slightly dilated RER, Golgi apparatus and mitochondria. In all 6 animals treated with 10°C HCA, the structure of the cytoplasmic organelles was intact, with no apparent dilatation (p=0.015).

Conclusion: This study adds further support that hypothermia at 10°C exerts better cellular protection than hypothermia at 18°C, as evidenced by these electron microscopy findings.

INTRODUCTION

Hypothermic circulatory arrest (HCA) is routinely used to interrupt normal perfusion of the brain and prevent subsequent cerebral ischemic injury during various cardiovascular procedures. Despite its widespread use, persisting concerns remain regarding the optimal brain temperature for its implementation. Experimental studies have demonstrated that prolonged HCA can lead to neuronal cell death. Research has focused on

studying the mechanisms leading to neuronal injury and eventual nerve cell death. Today, it is believed that apoptosis may contribute to the neuronal degeneration observed. Selective vulnerability occurs in the adult and neonatal brain and reflects heightened sensitivity of specific neuron groups to ischemic injury.¹ We have previously reported that the sensory and motor neocortex, and hippocampus are selectively vulnerable to injury in an acute model of HCA at 18°C; with the sensory cortex demonstrating the most notable neurologic injury compared to all neuronal populations.² Neurological damage in these areas may explain, in part, impairment of memory, cognition and motor function seen in adults after cardiac arrest.¹

Hypothermia is essential for cerebral protection during HCA. Hypothermia reduces cerebral metabolic activity,

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oxygen demand, prevents the release of neurotransmitters and delays the onset of a fatal biochemical cascade.³ Although reduced, brain metabolism is not suppressed adequately and remains relatively high at 18°C in traditional HCA protocols.⁴ In light of evidence suggesting that the apoptotic pathway may be reversible in their earlier stages,⁴ the present study was undertaken to assess whether cooling to 10°C can reduce neurological injury during 75 minutes of HCA in an acute porcine model compared to less profoundly cooled (18°C) animals as assessed by ultra structural changes in the sensory cortex.

MATERIALS AND METHODS

Twelve male juvenile pigs from a commercial farm, 2-3 months of age and weighing 25-35 Kg were used for this study. The animals were divided into 2 groups: Group A ($n=6$) underwent hypothermic circulatory arrest at 18°C for 75 min, and Group B ($n=6$) underwent hypothermic circulatory arrest at 10°C for 75 min. All animals were treated in accordance with the Principles of Laboratory Animal Care, as described by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. The protocol used in this study was also approved by the Animal Care and Use Committee of the University of Ioannina.

Preparation and surgery were performed as previously described.² Briefly, catheters were inserted in an ear vein and the left femoral artery for monitoring purposes and blood sampling. Anesthesia was induced with intramuscular ketamine hydrochloride (15 mg/kg), atropine (0.05 mg/kg), and midazolam (0.1 mg/kg) and was maintained with intravenous fentanyl (50-200 µg/kg), midazolam and 1% to 2% isoflurane. Muscle relaxation was achieved with a bolus intravenous rocuronium (0.6 mg/kg) and was maintained with 20% of the total dose every 30 min. Animals were ventilated mechanically with 100% oxygen, after endotracheal intubation. Ventilator rate and tidal volume were adjusted to maintain the arterial carbon dioxide tension at 40 mmHg. Hematocrit values during cardiopulmonary bypass (CPB) were maintained between 13%-23%. A temperature probe was placed in the rectum, while brain temperature was determined with bilateral tympanic membrane probes.

The chest was opened via a right thoracotomy in the fourth intercostal space. After administration of intravenous heparin (300 IU/kg), cannulas were advanced to the ascending aorta and to the right atrium. Non-pulsatile CPB, was initiated at a flow rate of 100 ml/kg per min and then adjusted to maintain a minimum arterial pressure of 50 mmHg. CPB was continued for an average of 58 or 106 minutes, to reach a deep brain temperature of 18°C or

10°C, respectively. Myocardial protection was afforded by applying iced saline (4°C) topically during the 75-minute interval of hypothermic circulatory arrest. When the tympanic membrane temperature reached 18°C or 10°C, bypass was discontinued, the blood was drained into the oxygenator reservoir, and circulatory arrest was maintained for 75 minutes. Ice bags were positioned around the head to maintain the brain temperature during HCA. At the end of HCA, bypass was initiated again with gradual rewarming to a rectal temperature of approximately 35°C to 36°C. A temperature of 36°C was reached after an average of 83 or 104 minutes of reperfusion for animals treated with 18°C or 10°C HCA, respectively. Systemic pressure was maintained above 60 mmHg during reperfusion.

At the end of the experiment, brains were perfused *in situ* with chilled saline solution 0.9% (1L) followed by 4% paraformaldehyde in 0.1mol/L phosphate-buffered saline solution (1L, pH7.4). The descending aorta was cross-clamped to avoid significant loss of perfusion solution to the lower body. The brains were removed *in toto*, immersed in 4% paraformaldehyde, and stored at 4°C in phosphate-buffered saline solution.

All brains were dissected in the sagittal plane. Tissue blocks from the left hemisphere were cut to encompass the postcentral gyrus (sensory neocortex). Tissue blocks were dehydrated in ethanol and xylene and embedded in paraffin. Serial 8-µm sections were cut from each tissue block and were mounted onto slides. Hematoxylin and eosin was used to characterize cell damage morphologically.

Paraffin embedded samples were dewaxed in xylene. After rehydration using graded ethanol, slices were washed in cold 0.1 M sodium cacodylate buffer and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight. Samples were washed in cold cacodylate buffer and then post-fixed with 1% osmium tetroxide in the same buffer for 1 hr at room temperature. After osmium tetroxide treatment, the samples were washed with 0.1 M sodium cacodylate buffer.

The selected area was identified with a dissecting microscope, and 2x2x2 mm sections were cut out from the coronal slices and dehydrated in a graded series of ethanol, before being embedded in epoxy resin. Blocks were trimmed, and semithin 0.5-µm sections were cut with an ultramicrotome and stained with toluidine blue for light microscopy analysis. Ultrathin (75-90 nm) sections were cut and placed on 200-mesh copper grids for double-staining with uranyl acetate and lead citrate. Samples were examined with a JEOL JEM 100 CX-II electron microscope.

Samples were examined in a blind fashion by 2 investigators using a JEOL 100CX electron microscope. Investigators

identified 10 representative neurons (per experimental brain specimen), according to the presence of a typical nucleus and surrounding perikaryon, and analyze electron micrographs of these neurons for evidence of nuclear changes (including chromatin dispersion or clumping), cytoplasmic changes, overall shape of the neuron (whether it was shrunken or swollen) and the appearance of rough endoplasmic reticulum (RER) compared with matched controls. Similarly, each investigator was instructed to examine the perinuclear neuronal mitochondria for abnormalities in mitochondrial distribution or shape, matrix density, crystal structure, and appearance of any abnormal structures; each finding was indicated as present or absent. In case of discrepancy, this was solved by consensus.

Values are expressed as mean \pm standard deviation (SD) unless otherwise indicated. When appropriate, differences between two groups were assessed by Fisher's exact test. A difference of <0.05 was considered to be significantly important.

RESULTS

All experimental animals survived the surgical protocol and HCA. Baseline variables and cooling-rewarming duration data are shown in Table 1. No hemodynamic differences were observed in the 2 groups. No evidence of neuronal injury in the sensory cortex was observed in any of the specimen assessed using plain histology with hematoxylin and eosin staining.

Electron microscopic observations in the present study provided no robust morphological evidence to confirm apoptosis or necrosis of the sensory cortical neurons in model of HCA. In general, neurons showed normal nuclear and cytoplasmic morphology in both treatment groups, with only minor ultra structural changes in cellular organelles observed after HCA at 18°C. There was no evidence of cell swelling. No samples of either group displayed evidence of change regarding the overall shape of the neuron and nucleus. Neurons exhibited pale round or oval nuclei. Nucleoli were intact and plasma and nuclear membranes remained intact.

Significant differences were observed between the 2 groups in the cell organelles, namely mitochondria and RER.

Table 1: Baseline variables and cooling-rewarming duration data

	Group A	Group B
Weight (kg)	30.6 \pm 3.7	34.2 \pm 3.1
Age (days)	70.5 \pm 7.7	86.2 \pm 5.6
Cooling duration (min)	57.5 \pm 17.2	105.8 \pm 21.8
Rewarming duration (min)	82.5 \pm 10.4	104.2 \pm 19.8

In 5 of the 6 animals treated with 18°C HCA (Group A), neurons had slightly dilated RER, Golgi apparatus and mitochondria (Figures 1a and b). In some cases, polysomes were disassociated, displaying desegregated ribosomes. Although some mitochondria were dilated, they showed an otherwise normal morphology (Figure 2). In all 6 animals treated with profound hypothermia (10°C HCA; Group B), the structure of the cytoplasmic organelles was intact, with no apparent dilatation ($p=0.015$).

DISCUSSION

Hypothermia is the only therapeutic modality that has been shown to exert favorable results in HCA,⁵ even if these are incomplete.⁶ However, this intervention does not completely halt the neurologic injury that is usually present in HCA. Recent studies using a variety of methods have noted multiple different patterns of cell damage in brains following HCA.^{2,7,8}

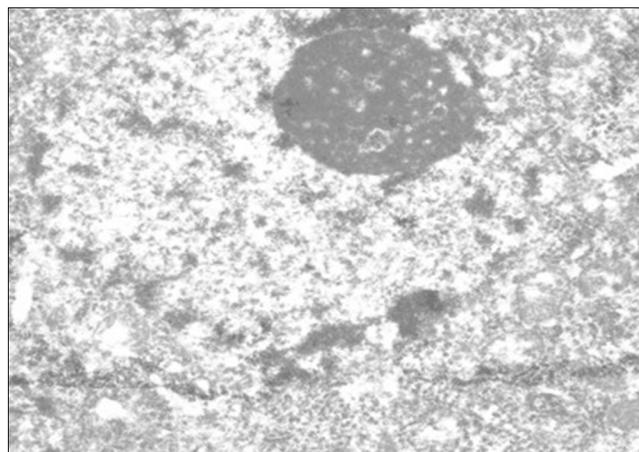


Figure 1a: Sensory cortex from HCA at 18°C (group A). Electron micrograph following HCA at 18°C with detail of neuron, showing nucleus (x18400)

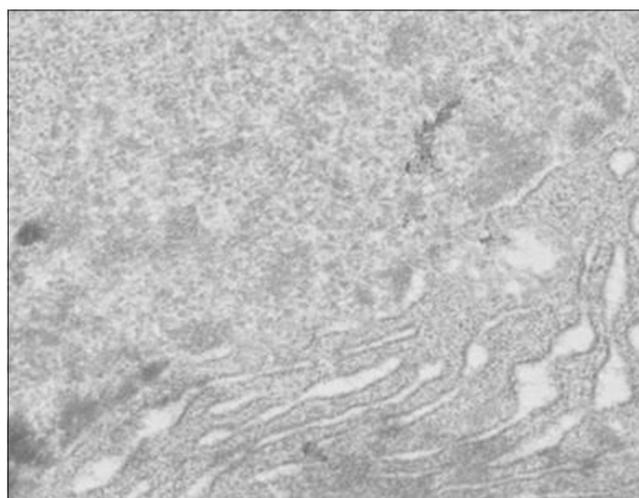


Figure 1b: Sensory cortex from HCA at 18°C (group A). The same neuron showed dilated rough endoplasmic reticulum (x25200)

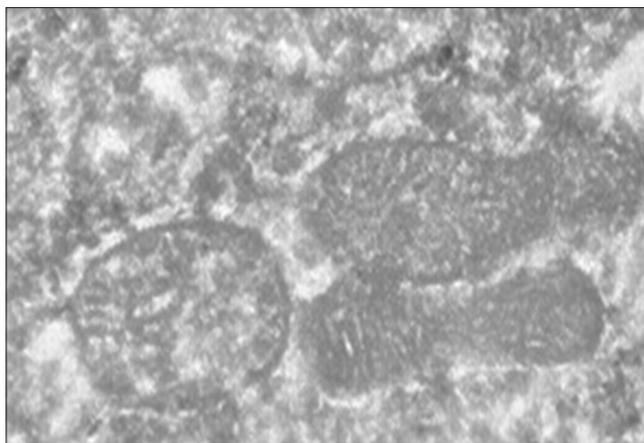


Figure 2: Neurons from the sensory cortex of animals treated with HCA at 18°C also exhibited slightly dilated mitochondria (m) as seen here. (x 46000)

The protective effects of hypothermia may be attributed to the fact that the method protects cells from damage by retarding the rate of energy depletion during the ischemic phase.⁹ Hypothermia has been found beneficial in preserving cerebral energy metabolism, which in turn results in delayed anoxic depolarization.¹⁰

Previously, we assessed acute neuronal injury in various regions of the brain after HCA at 18°C in a porcine animal model. We reported that neurons in the sensory and motor neocortex, as well as in the hippocampus were vulnerable to cell injury acutely after 75 minutes of HCA, as determined by a positive TUNEL reaction for DNA-fragmentation.² Although nerve cell populations in the cerebellum, thalamus and ventral medulla also showed cell injury, the percentage of TUNEL positive cells in these areas was significantly less than that observed in the primary motor and sensory cortex, and in the hippocampus.² TUNEL (+) staining was significantly less at 10°C in the motor and sensory cortex and the hippocampus compared to 18°C HCA, indicating that there was increased cerebral protection.¹¹ The observation that TUNEL labeled cells may eventually, but not necessarily, progress into morphologically distinct apoptotic cells also confirms the idea that different morphologic characteristics may reflect different stages of the same death process.

This premise is supported by our electron microscopy findings. At 18°C HCA, the neurons of the sensory cortex displayed dilation of the rough endoplasmic reticulum and mitochondria, detachment of ribosomes, along with Golgi derived vacuolation. These findings suggest that the sensory cortex was in Phase II of the apoptotic process.¹² At 10°C hypothermia, the ultra structural findings suggest that the sensory cortex was in Phase I, showing only mild dilation of the rough endoplasmic reticulum and detachment of ribosomes. Although both groups appear to be in earlier stages of apoptosis, the findings clearly indicate that HCA at

18°C is associated with more morphological characteristics of apoptosis, compared to 10°C.

Most previous studies use the classic 90-minute HCA, 20°C model, which results in more severe cerebral injury with the appearance of morphologically characterized apoptotic cells at approximately 8 hours following HCA.^{1,7} In the present acute model, animals were treated with HCA for 75 minutes, and were evaluated after approximately 83 - 104 minutes of reperfusion. Normal cell suicide programs demonstrate a rapid cell death, which kill a cell within 2 to 3 hours. Also, more subtle injuries appear to result in a greater proportion of damaged cells being shunted into apoptosis. As such, earlier chronic models may have underestimated the contribution of apoptosis to the cerebral sequelae after HCA.

This is a follow-up study to previous investigations by the authors designed to assess a possible mechanism of neuronal injury. Taken together, the studies demonstrate that our model addresses a very early point of activation of the apoptotic pathway. Although this model does afford an early examination of apoptotic triggers and a model system for further investigation at the genomic and proteomic level, it does not provide robust morphologic end-point evidence of cell death. The model of 10°C is primarily a “proof of concept” system. This extreme form of hypothermia has been demonstrated in both animal models, as well as isolated clinical scenarios, to be detrimental to other end organs, such as the lung. There remain several important gaps in how to best study cerebral outcome following HCA (e.g. clinically relevant animal model, interval of arrest, depth of hypothermia, rewarming, optimal perfusion characteristics, etc.) As a consequence, most laboratory findings are diluted in translation, resulting in a laboratory-clinical divide that hinders progress towards optimal clinical application. Further studies are warranted to address the clinical significance of the present findings.

In conclusion, this study adds further support that hypothermia at 10°C exerts better cellular protection than hypothermia at 18°C, as evidenced by these electron microscopy findings.

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