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Neurologic Injury Following Hypothermic Circulatory Arrest

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1. Introduction

Cardiothoracic surgeons are faced with the challenge of protecting the brain during the sensitive time of interruption of normal cerebral blood flow. The brain is an exceptionally complex organ with a functional anatomy that is difficult both to understand and assess. Experimental and clinical studies have shown that the mechanism of neural injury is multifactorial. As such, discussions regarding the best surgical strategies for neuroprotection during circulatory arrest are formidable, at best. Although we are armed with excellent experimental and clinical studies that demonstrate the deleterious effects of prolonged exposure to cardiopulmonary bypass (CPB) on brain function and structure, the various neuroprotective strategies, particularly that of deep hypothermic circulatory arrest (DHCA) remain an issue of debate. This is related in part to the gap between the basic science understanding of brain injury caused by these events and the clinical application of various neuroprotective strategies and their subsequent clinical outcomes. The goal here is to address the current understanding of the mechanisms underlying brain injury after HCA and relevant strategies of neural protection, supported by primary experimental data from our laboratory.

2. Hypothermic circulatory arrest

The use of therapeutic hypothermia dates back to the ancient Egyptians, Greeks, and Romans. In modern times, the use of therapeutic hypothermia progressed from observation case reports to animal studies to clinical use in children and then adults. Initially there were observational reports of therapeutic use of hypothermia in patients with severe cerebral trauma, followed by experimental studies in dogs that suggested a therapeutic role for hypothermia for cerebral protection during cardiac surgery. Later, profound hypothermia (12º C, nasopharyngeal) with circulatory arrest (up to 1 hour) was used in children undergoing surgical repair of the tetralogy of Fallot. (Apostolakis & Shuaiber, 2007) The use of deep hypothermic cardiopulmonary arrest (DHCA, 14-18°C) was first applied as a method for cerebral protection during the prosthetic replacement of the aortic arch.(Griepp et al, 1997) Later, the use of a DHCA was extended into other major vascular
surgeries such as the repair of thoracoabdominal aortic lesions, clipping of giant and complex cerebral aneurysms, and resection of renal carcinoma with tumor thrombus extending into the inferior vena cava or atrium.

Deep hypothermic circulatory arrest (DHCA or HCA) provides 2 clinical benefits. The circulatory arrest component provides a bloodless surgical field without the need for the use of intrusive clamps and cannulae. The deep hypothermic component significantly decreases brain metabolism and oxygen requirements and thus permits a longer period of interrupted blood perfusion to the brain. The cerebral metabolic rate is related exponentially to brain (core body) temperature, with the cerebral metabolic rate decreasing by about 50% for each 6°C drop in brain temperature. Since the first experimental studies, the use of DHCA has become the standard technique for the surgical repair of certain congenital and acquired cardiovascular lesions. The outcome after these operations improved considerably over the past two decades and surgery requiring HCA can usually be performed with an acceptable risk for the patient. However, it is likely that these improvements are more a consequence of an increasing expertise with this type of surgery, rather than the influence of one particular organ protection method employed. Despite this fact, there is still room for improvement, since prolonged periods of HCA are still associated with significant morbidity and mortality. As the brain is the organ most sensitive to ischemic damage, it is considered to be the limiting factor for the duration of HCA. Nevertheless, despite its protective effects, HCA can be detrimental for other organ systems.

The basic established techniques and perfusion strategies during aortic arch replacement number three: hypothermic circulatory arrest (HCA), antegrade cerebral perfusion (ACP), and retrograde cerebral perfusion (RCP). During the past decade and after several experimental studies, RCP lost its previous place in the armamentarium of brain protection, giving it up to ACP as a major method of brain perfusion during HCA. HCA should be applied at a temperature of ≈20°C with long-lasting cooling and rewarming and should not exceed by itself the time of 20–25 min. RCP does not seem to prolong safe brain-ischemia time beyond 30 min, but it appears to enhance cerebral hypothermia by its massive concentration inside the brain vein sinuses. HCA combined with ACP, however, could prolong safe brain-ischemia time up to 80 min. Cold ACP at 10°–13°C should be initially applied through the right subclavian or axillary artery and continued bihemispherically through the left common carotid artery at first and later the anastomosed graft, with a mean perfusion pressure of 40–70 mm Hg. The safety of temporary perfusion is being confirmed by the meticulous monitoring of brain perfusion through internal jugular bulb O₂ saturation, electroencephalogram, and transcranial comparative Doppler velocity of the middle cerebral arteries (Kouchoukos et al, 2003).

3. Methods of end-organ protection during DHCA

3.1 Hypothermia

Hypothermia acts by reducing the metabolic rate of the brain and improving the balance between energy supply and demand. Hypothermia reduces cerebral blood flow (CBF) in a linear manner, but the decrease in cerebral metabolic rate of oxygen (CMRO₂) is not exactly linear. On average, the reduction in CMRO₂ is about 7%/1°C. Between 37°C and 22°C, CMRO₂ is reduced by about 5%/1°C, and then the reduction accelerates when CMRO₂ reaches 20% at 20°C and 17% at 18°C, at which point about 60% of patients achieve electrical silence on electroencephalography (EEG).
3.2 Drug interventions
To increase the tolerance to ischemia, the use of potentially neuroprotective drugs is an appealing concept, especially since the circulatory arrest interval is well defined and allows a preischemic treatment. Therefore, it is evident that the use of these pharmaceuticals is more promising in HCA patients than for the postischemic treatment of patients after embolic strokes. Studies in a chronic porcine model showed that nontoxic drugs are available that have neuroprotective effects, making them potential candidates for clinical use. Additionally, combining drug treatment with selective perfusion techniques, to support adequate delivery of the agent into the target organ, seems to be a promising concept.
Among the various neuroprotective pharmacologic agents are barbiturates, which are believed to be protective in focal ischemia by reducing CMRO$_2$, CBF, free fatty acids, free radical and cerebral edema. Steroids decrease proinflammatory responses, while beta-blockers decrease the inflammatory response. Mannitol reduces cerebral edema, scavenges free radicals and protects the kidneys by lowering renal vascular resistance. Furosemide blocks renal reabsorption of sodium, and insulin controls hyperglycemia, which in turn prevents intracellular acidosis. Lidocaine is a selective blocker of Na$^+$ channels in neuronal membranes and thus, reduces CMRO$_2$. Dexmedetomidine inhibits ischemia-induced norepinephrine release and is protective for both focal and global ischemia. Acadesine appears to mitigate the effects of reperfusion injury.

3.3 Intraoperative neuromonitoring
Neurophysiological monitoring during thoracic aortic surgery using HCA became increasingly popular in the last decade. Besides its value during an ongoing operation, the collection of data in combination with outcome analysis might help to improve or change surgical strategies. Continuous recording of electroencephalograms (EEGs) as well as SSEPs is now routine in most neurosurgical units. The use of neuromonitoring in cardiothoracic surgery is in part hampered by the fact that hypothermia has an impact on the sensitivity of neurophysiological measures, so they cannot be used during deep hypothermia. On the other hand, some surgeons have found this an asset, and use disappearance of the EEG to determine the optimal level of hypothermia before they stop the extracorporeal circulation. Therefore, the value of the EEG as an isolated method for ascertaining whether cerebral protection is adequate is questionable. Furthermore, nonsynaptic metabolic activity may persist even when the EEG is isoelectric. On the other hand, the EEG may provide valuable information for those groups which are using relative high blood temperatures during SCP. Furthermore, EEG seems to be a good tool for detecting electrophysiological recovery in the early postoperative period. Monitoring of SSEPs is generally easier than EEG since electric noise does not play such a substantial role. It is generally less influenced by anesthetic drugs, and it remains detectable as long as cortical activity can be encountered. From clinical experience, SSEPs seem especially valuable during surgery on the descending or thoracoabdominal aorta (which is not subject of the present synopsis) but muscle evoked potentials (MEPs) may be even more sensitive for detection of spinal cord injury.

3.4 Acid-base management during hypothermia
Hypothermia alters the results of analysis of arterial blood gases by increasing the solubility of CO$_2$ and O$_2$ in plasma. The increase in CO$_2$ solubility decreases the concentration of the insoluble portion and, thus, the partial pressure. However, the total content of CO$_2$ in the
blood remains the same. During hypothermia, if a blood sample is taken and warmed to 37°C in the blood gas analyzer, the CO₂ initially dissolved will now contribute to the partial pressure of CO₂ (PCO₂) and the PCO₂ will be within the normal normothermic range. If, on the other hand, the value is estimated at the patient’s actual temperature, the PCO₂ will be reduced despite similar arterial CO₂ content. In addition to its effect on gas solubility, hypothermia decreases the metabolic rate and CO₂ production. Maintaining the PCO₂ within the normal range in rewarmed 37°C blood is called “alpha-stat.” If the PCO₂ is corrected to the patient’s actual temperature and that value is kept within the normal range, the management is called “pH-stat.”

4. Duration of DHCA

A number of biochemical and cellular structural changes take place as the duration of circulatory arrest lengths. After 15 minutes of ischemia at 18°C, the recovery of oxygen consumption is impaired, and after 20 minutes, cerebral lactate is detected in the effluent blood. The safe duration of circulatory arrest at 15°C was predicted to be about 29 minutes and at 10°C about 40 minutes. If ischemic tolerance is considered 5 minutes at normothermia, the calculated safe period of circulatory arrest at 18°C would be 15 minutes. Clinical studies have shown a persistent loss of cognitive function (lasting more than 6 weeks) and deterioration in postoperative cognitive scoring/testing in patients who underwent aortic arch surgery by using DHCA for more than 25 minutes at 10°C.

5. Complications of DHCA

Disadvantages of DHCA include increased cardiopulmonary bypass (CPB) time, edema formation, coagulopathy, and alteration in many organ functions including the kidney, the brain, vascular smooth muscles, intestinal mucosa, alveolar epithelium, the liver, and the pancreas. Based on reports from 8 major cardiac surgery centers in the United States, Europe, and Japan, the risk of permanent neurologic injury after aortic arch surgery using DHCA ranged from 3% to 12%, renal dysfunction from 5% to 14%, pulmonary insufficiency from 5% to 39%, and left ventricular failure or low-cardiac-output syndrome from 7% to 34%. Alternatives to the use of DHCA during aortic arch replacement are the use of normothermic CPB or mild-to-moderate degrees of hypothermia. These alternatives obviously require the use of a perfusion system for the brain, separate from the rest of the body, which might increase the risk of cerebral embolization.

5.1 Neurologic injury

Neurologic injury is the most troublesome adverse effect of DHCA and CPB, presenting either as transient neurologic deficit (5.9%-28.1%) or irreversible neurologic injury (1.8%-13.6%). Early postoperative mortality markedly is increased (18.2%) in patients with neurologic injury, and long-term cognitive disability is common among survivors. Neurologic deficit after DHCA encompasses a wide scale of disorders ranging from deep coma to subtle, hardly perceptible alterations in cognitive functions or behavior. In the immediate postoperative period, the return of sophisticated neurologic functions is often obscured by the administration of sedative and analgesic agents. Neurologic injury presents at that time mostly as a focal or diffuse deficit. A focal deficit is due to interruption of blood in a terminal vascular territory, usually following embolism of material or gas bubbles. The
clinical expression is typically motor-sensory deficit, aphasia, or cortical blindness (Kunihara et al, 2005; Lipton, 1999). Computed tomography and magnetic resonance imaging are usually able to detect a sharply demarcated area of necrosis in the brain. A focal deficit is usually an embolic phenomenon, whereas a prolonged poor perfusion of the brain may produce necrosis in watershed zones. Age, atherosclerosis, and manipulation of the aorta are risk factors for both. Global cerebral ischemia leads to diffuse neurologic deficit, which may be benign and reversible or more debilitating (seizures, Parkinsonism, and coma). Risk factors include increased duration of circulatory arrest and CPB, diabetes mellitus, and hypertension. Transient neurologic dysfunction appears to be a marker of long-term cerebral injury. Deficits of memory and fine-motor function may persist after hospital discharge. Reductions in CMRO\textsuperscript{2} and the duration of DHCA reduce the risk of neurologic injury. The length of time on CPB might be a better predictor of postoperative death and stroke than the duration of DHCA time (Hagl et al, 2003).

Aortic procedures requiring hypothermic circulatory arrest have been specifically correlated with increased risk of both stroke and mortality in all patients. This may be accentuated in the elderly, who may have less tolerance for neurological insult. Many physicians think patients >75 years old are too frail and lack the reserve to survive a major cardiothoracic surgery. In particular, there remains some hesitancy in performing procedures with a higher risk of stroke in patients with a higher susceptibility for adverse neurological sequelae. This perceived combination of risk and susceptibility may be a barrier to care for elderly patients requiring hypothermic circulatory arrest to address their aortic pathology. According to Coselli et al. (2008), in a study accessing the safety and efficacy of HCA, there are various major complications associated with HCA. These included death (interoperative, during hospital stay, and within 30 days), stroke, paraplegia, paraparesis, uncontrolled bleeding which required reoperation, renal failure, cardiac complications, and vocal cord paralysis.

6. Strategies for brain protection during DHCA

6.1 Hypothermia – reduction of metabolism

Hypothermia is the most efficient measure to prevent or reduce ischemic damage to the central nervous system when blood circulation is reduced. The central nervous system has a high metabolic rate and limited energy stores, which make it extremely vulnerable to ischemia (Elrich et al, 2002). Hypothermia acts by reducing the metabolic rate of the brain and improving the balance between energy supply and demand, and thus lengthens the period of tolerated ischemia. Hypothermia reduces cerebral blood flow (CBF) in a linear manner, but the decrease in cerebral metabolic rate of oxygen (CMRO\textsuperscript{2}) is not exactly linear. On average, the reduction in CMRO\textsuperscript{2} is about 7%/1°C. Between 37°C and 22°C, CMRO\textsuperscript{2} is reduced by about 5%/1°C, and then the reduction accelerates when CMRO\textsuperscript{2} reaches 20% at 20°C and 17% at 18°C, at which point about 60% of patients achieve electrical silence on electroencephalography (EEG) (McCullough et al, 1999).

6.2 Techniques and perfusion strategies

Although reduction of cerebral metabolism and swift surgery are the two fundamental measures that can prevent or reduce brain damage during circulatory arrest, there are adjunctive protective measures that can be considered. The basic established techniques and perfusion strategies during aortic arch replacement number three: hypothermic circulatory arrest (HCA), antegrade cerebral perfusion (ACP), and retrograde cerebral perfusion (RCP).
7. Experimental investigation of cerebral injury following DHCA

A clearer understanding of the consequences of HCA will be pivotal in clinical decision-making, including when to initiate circulatory arrest and the appropriate interval. Delayed cell death is of special interest because of the potential for intervention. Although apoptosis is believed to play a part in the cerebral injury, its role has generally been identified through histologic techniques. These snapshots do not permit a clear delineation of the time-line of apoptosis. Because its role is not clear, therapies have yet to be designed for the specific purpose of inhibiting apoptosis.

The balance between cell survival and death is under tight genetic control (Almeida et al., 2000). A multiplicity of extracellular signals and intracellular mediators are involved in maintaining this balance. When the cell is exposed to physical, biochemical or biological injury, or deprived of necessary substances, it activates a series of stress-response genes. Although with minimal insults, the cell may recover, with greater insults, single cell death results. The current understanding of the neurons response to insult has been supported by evidence from a series of studies using a porcine model system to investigate the effects of hypothermic circulatory arrest and ischemic insult on the integrity of neuronal populations.

7.1 Clinically relevant animal models

Evaluating various strategies and treatments in animal studies in order to determine clinical feasibility remains a challenge. Animal models have contributed immensely to our understanding of cerebral consequences of HCA, with several animal models having been used. To date, the preclinical investigation of cerebral injury mechanisms related to deep hypothermic circulatory arrest has been limited to large-animal models (porcine, canine and ovine). These models are expensive, personnel demanding, cumbersome and are usually performed without validated neuropsychologic assessment. Rodent models have been attempted to overcome some of these disadvantages, although treatment effects cannot always be confirmed in the rat model. Ultimately, however, each experimental model system from cell cultures to rats, to large animals and ultimately to clinical trials, have their advantages and disadvantages, and ultimately their place in these investigations.

Most animal models require an extended period of arrest to produce a reproducible level of neuronal injury that would facilitate elucidating the mechanisms of injury and efficacy of neuroprotective interventions. Many large animal models require DHCA for at least 90-120 minutes to demonstrate neurologic deficits. Although such prolonged DHCA interval might not be considered clinically realistic, they may be more appropriate for demonstrating the molecular pathways behind acute neuronal injury and hence, modes of intervention (Conti et al, 1998).

Study of a neuroprotective strategy includes appropriate selection of an animal model and functional indices. The model is selected with respect to their relevance and feasibility of assessing the parameters of interest. Investigations of promising neuroprotective methods require validation (validation study), use in experimental settings to optimize cerebral protection during CPB and DHCA (performance study) and test during routine cardiac surgery (clinical study).

Hypothermia is essential for cerebral protection during HCA. Hypothermia reduces cerebral metabolic activity, oxygen demand, and prevents the release of neurotransmitters and delays the onset of fatal biochemical cascade (Elrich et al., 2002; McCullough et al., 1999). Although reduced, brain metabolism is not suppressed adequately and remains
relatively high at 18°C in traditional HCA protocols (McCullough et al., 1999). In light of evidence suggesting that the apoptotic pathway may be reversible in their earlier stages (McCullough et al., 1999), studies from our team were 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 DNA fragmentation, anti-apoptotic protein Bcl-2 expression, and ultrastructural changes in the sensory cortex. Sixteen 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 three groups: Group A (n=6) underwent hypothermic circulatory arrest at 18°C for 75 min, Group B (n=6) underwent hypothermic circulatory arrest at 10°C for 75 min and Group C (n=4) served as normal controls.

Preparation and surgery were performed as previously described (Ananiadou et al 2005). Briefly, catheters were inserted in an ear vein and the left femoral artery for monitoring purposes and withdrawal of blood samples. Anesthesia was induced with intramuscularly ketamine hydrochloride (15 mg/kg), atropine (0.05 mg/kg), and dormicum (0.1 mg/kg) and was maintained with intravenous fentanyl (50-200 μg/kg), dormicum and 1% to 2% isoflurane. Paralysis 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. Urine output was collected through a bladder catheter (Foley 8-10 F). Arterial pressure, end-expired carbon dioxide, electrocardiogram, and blood gases (ABL Radiometer Medical A/S DK-2700, Copenhagen, Denmark) were monitored.

As previously described, the chest was opened via a right thoracotomy in the fourth intercostal space (Ananiadou et al., 2005). After administration of intravenous heparin (300 IU/kg), cannulas were advanced to the ascending aorta (16 F arterial cannula) and to the right atrium (single 26 F cannula). 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. To avoid distortion of the left ventricle during CPB, a 10 F vent catheter was inserted via the superior pulmonary vein. The lungs were allowed to collapse after CPB was initiated. The CPB circuit was primed with a bloodless solution consisting of 1000cc lactated Ringer’s, 50 ml mannitol, and 5000 IU heparin. Sodium bicarbonate was added to adjust the pH to 7.4, as necessary.

CPB was continued for an average 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 the arrest, bypass was initiated again with gradual rewarming to a rectal temperature of approximately 35°C to 36°C. A temperature gradient exceeding 10°C between the perfusate and the core temperature was avoided. 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. Measurements of hemodynamics (heart rate, mean arterial pressure), arterial blood gases, hematocrit, glucose lactate, as well as temperatures were recorded at five time points during the experiment. These were: 1) Baseline at 37°C and prior to CPB; 2) At the initiation of CPB; 3) During CPB, while cooling to a brain temperature of 18°C or 10°C just before HCA; 4) During rewarming; and 5) At the end of CPB.

The mean duration (+SD) of CPB cooling and CPB warming for animals with 18°C HCA was 57.50±17.25 and 82.50±10.37 minutes, and for 10°C was 105.8±21.8 and 104.2±19.8 minutes, respectively. Perioperative physiological variables are shown in Table 1. Although there were some minor variations, no apparent clinically relevant hemodynamic differences were observed between treatment groups. Lactate levels were significantly higher following HCA at 10°C compared to 18°C. PO2 levels were significantly lower in 18°C HCA animals compared to 10°C during cooling, and hematocrit levels dropped to a similar degree in all experimental animals during the procedure.

Table 1. Typical Physiologic Variables During HCA Paradigm in a Porcine Model
All values are expressed as mean ± SD. *P<0.05 between animals treated with HCA at 18°C vs 10°C (unpaired, 2-tailed, t-test). **P<0.05 between sample times (ANOVA followed by Fisher PLSD).
8. Neuronal injury and nerve cell death: basic sciences

Neuronal death is normal during development of the nervous system, but it is abnormal in brain and spinal cord disease and injury. The available evidence indicates that the survival of neurons and their death are highly regulated and finely orchestrated dynamic events that depend on a number of internal and external factors. Two types of cell death are recognized: cell necrosis resulting from injury and causes inflammation and apoptosis, observed normally in development and now identified as programmed cell death. Apoptosis and necrosis are types of cell death. They are generally considered to be distinct forms of cell death, but there is mounting evidence supporting an apoptosis-necrosis cell death continuum (Portera-Calliau, et al, 1997). In this continuum, neuronal death can result from varying contributions of coexisting apoptotic and necrotic mechanisms, resulting in some of the distinctions between apoptosis and necrosis becoming blurred. Today it is believed that apoptosis may contribute to the neuronal degeneration in neurological injuries such as cerebral ischemia and trauma (Kerr et al 1972; MacManus and Linnik, 1997; Martin, 2001).

Necrosis can result from acute oxidative stress characterized by passive cell swelling, rapid energy loss, and generalized disruption of internal homeostasis with lysis of the nucleus, intra-nuclear organelles and plasma membranes leading to the release of intracellular components that induce a local inflammatory response that in turn, result in edema and injury to neighboring cells. Morphologically, cell death is characterized by swelling of organelles and rupture. Necrotic cell death is characterized by inflammation and widespread damage.

Apoptosis is a process of cell suicide, the mechanisms of which are encoded in the chromosomes of all nucleated cells. Although the capacity to carry out apoptosis appears to be inherent in all cells, the susceptibility to apoptosis varies markedly and is influenced by external and cell-autonomous events. Apoptosis is regulated by complex molecular signaling systems resulting in an orderly, energy-dependent enzymatic breakdown into characteristic molecular fragments, DNA, lipids and other macromolecules. Apoptosis can be induced by cell surface receptor engagement, growth factor withdrawal and DNA damage. In contrast to those observed in cell necrosis, the morphological changes that occur during developmental cell death include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. Earlier studies showed that one of the biochemical hallmarks of apoptosis is DNA cleavage at internucleosomal linker regions, resulting in ladder formation of DNA of 180-200 bp or multiples thereof. However, this ladder-type DNA fragmentation is also found in some cells dying of necrosis, indicating that DNA fragmentation cannot be the sole criterion, but simultaneous morphological assessment must also be done for identifying apoptosis.

8.1 Mechanisms of apoptotic cell death

Several families of proteins and specific biochemical signal-transduction pathways regulate cell death. Cell death signaling can involve plasma membrane death receptors, mitochondrial death proteins, proteases, kinases and transcription factors. Predominant factors in cell death and cell survival include fas receptor, Bcl-2 and Bax (and their homologues), cytochrome c, caspases, p53 and extra cellular signal-regulated protein kinases. Some forms of cell death require gene activation, RNA synthesis and protein synthesis, whereas other forms are transcriptionally-translationally independent and are...
by posttranslational mechanisms such as protein phosphorylation and protein translocation.
The precise signaling cascade starting from the detection of the signal at the cell surface to
the events that occur in the nucleus in apoptosis is not well established, with several grey
zones in most suggested pathways. However, many events that occur at the cell surface and
intracellularly during apoptosis in the nervous system have been reported. Following an
appropriate stimulus, the first stage or “decision phase” of apoptosis is the genetic control
point of cell death. This is followed by the second stage or “execution phase”, which is
responsible for the morphological changes of apoptosis. The decision phase or genetic
control appears to be mediated by two genes Bcl-2 and p53, while the execution phase
appears to result from the activation of caspases. It has become apparent that the Bcl-2
family of proteins constitutes a critical intracellular checkpoint within a distal common
pathway of programmed cell death (Almeida et al, 2000).

8.2 Selective vulnerability of neural populations to neural insult after HCA
After assessing acute neuronal injury in various regions of the brain after HCA in a porcine
animal model, we found that neurons in the sensory and motor neocortex, as well as those
in the hippocampus, were vulnerable to cell injury acutely after 75 min of HCA at 18°C, as
determined by a positive TUNEL reaction for DNA fragmentation (Ananiadou et al, 2005).
TUNEL positive cells are identified by a red-stained, condensed nucleus with apoptotic
bodies, along with a diminutive or absent cytoplasm. (Figure 1) Although nerve cell
populations in the cerebellum, thalamus and ventral medulla were also found vulnerable to
cell injury, the percentage of TUNEL positive cells in these areas was significantly less than
that observed in the primary motor and sensory gray matter, and in the hippocampus.
(Table 2)

Fig. 1. Typical Presentation of TUNEL Positive Cells.
Photomicrograph showing apoptosis in the brain following HCA in an acute porcine model.
Cluster of TUNEL (+) apoptotic neurons (nucleus is red stained) are interspersed among
normal neurons in the anteroventral medulla. (magnification x400)
Age and temperature appear to influence neuronal injury, by making certain nerve cell populations more vulnerable to injury. In particular, the hippocampus, cerebellum, striatum, thalamus, amygdala and neocortex have been reported vulnerable in adult normothermic ischemia. In contrast, newborns were more vulnerable to injury in the neocortex and striatum. In the present model of hypothermic ischemia in juvenile pigs, the neocortex and hippocampus demonstrated the greatest vulnerability to insult during HCA. The apparent higher level of TUNEL positive cells in the primary sensory cortex (post-central gyrus) is not clear.

Although these previous studies clearly support that some of the cell death observed in HCA is via an apoptotic pathway, the experimental conditions used may underestimate the contribution of apoptosis to the cerebral sequelae after HCA (Kurth et al, 1999; Hagl et al, 2001). In this regard, some authors have expressed concern regarding the temporal pattern of brain damage and apoptosis after HCA. Thus, although recently improved methods of perfusion-fixation and more sophisticated analysis, have clearly shown the HCA initiates a series of events that ultimately leads to cell death via a typical apoptotic pattern, the time course of these events remains unclear. Most of these previous studies use the classic 90-min HCA, 20 °C model, which results in more severe cerebral injury than that usually observed clinically, where HCA is carried out for shorter intervals. The results from earlier studies also demonstrated that serious cell injury exists as early as 6 h after HCA, and that this process continues for at least 72h.

The importance of understanding the time course of events is underscored by an earlier study of long-term survivors of the 90-min, 20 °C protocol. Although treatment with CsA was reported to improve behavioral recovery after 7 days, at the 7 days time point after HCA there was no difference between CsA treated animals and controls for apoptosis measures. The authors concluded that they had missed the peak of apoptosis, and that an effective reduction in nerve cell injury would be found most likely with CsA treatment had they examined brain tissues at an earlier time point.

We found no morphological evidence of apoptosis or necrosis, but significantly greater levels of TUNEL positive cells in the brain regions assessed, compared to normal control animals. We hypothesize, that these findings indicate an early point of activation of the apoptotic
pathway. This is consistent with the rapid cell death observed in normal cell suicide programs that can kill a cell within 2 to 3 h. At an earlier time point, such as that in this study, we would not anticipate completion of the apoptotic mechanism, resulting in cell death with its classic morphological characteristics, but rather the initiation of the cellular response cascade. Certain cell populations appear to be more vulnerable to injury. In particular, the hippocampus, cerebellum, striatum, thalamus, amygdala and neocortex are more vulnerable in adult normothermic ischemia. In contrast, newborns are more vulnerable to injury in the neocortex and striatum. Our studies show that hypothermia does not provide equal protection to all regions of the brain. In the juvenile pig model, the neocortex and hippocampus demonstrated the greatest vulnerability to insult during HCA. The apparent higher level of TUNEL positive cells in the primary sensory cortex (post-central gyrus) is not clear, and demands further investigation. (Figure 2)

Fig. 2. Positive TUNEL Reaction in Vulnerable Neural Regions
Regional pattern of neuronal death after deep hypothermic circulatory arrest in juvenile pigs. Each point represents the mean score from six experimental animals for each brain region. The brackets indicate the S.D. Among the HCA treated animals, significantly higher concentrations of TUNEL (+) cells were observed in the sensory cortex, motor cortex and hippocampus, compared to the cerebellum, thalamus and medulla (P< 0.05 by ANOVA followed by Fisher PLSD). Although not statistically significantly greater than the motor neocortex and hippocampus, the postcentral gyrus had greater TUNEL scores compared to the medulla and thalamus (P<0.01). [*P<0.05 vs sensory and motor cortex, and hippocampus; ** P<0.001 vs sensory cortex]

8.3 Profound hypothermia reduces apoptotic neurological injury after HCA
The use of HCA in aortic repair and congenital heart surgery is based on the idea of reducing the metabolic rate and thus, allowing a more prolonged interval without perfusion that can be safely tolerated by the brain. The brain, in general, is very sensitive to hypoxia-
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ischemia because it has a high metabolic rate and small reserve of high-energy carbohydrates and phosphates. Several studies have indicated that cerebral metabolism is reduced effectively at profound levels of hypothermia, suggesting that protection of the brain should be greater when HCA is performed at even lower temperatures, such as that used in our studies (Strauch et al, 2005).

We have found that profound hypothermia at 10°C during HCA resulted in a significant reduction in neurological injury in selectively vulnerable brain regions. 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 (Ananiadou et al, 2008). These findings are compatible with previous reports that profound hypothermia results in a superior neurological outcome compared to conventional HCA methods. Although this study does not elucidate the mechanisms, it does affirm that profound hypothermia exerts a neuroprotective effect.

8.4 The decision phase for apoptotic nerve cell death: evidence for Bcl-2

Apoptosis is controlled genetically, and two genes, Bcl-2 and p53 are now believed to be important. It is now established that proteins encoded by the Bcl-2 gene family are major regulatory components of the apoptotic pathway (Kroemer, 1997). Within the apoptotic cascade, several proteins that facilitate neuronal survival compete with molecules that contribute to cell death. Ultimately, the final balance between cell survival-promoting proteins versus cell death-promoting proteins determines the fate of the cell. The Bcl-2 family of proteins plays an important role in this cell survival-cell death decision. This hypothesis is supported by our findings in Bcl-2 expression. The Bcl-2 family of proteins is important for the regulation of apoptosis during the “decision phase.” An increase of Bcl-2 has been suggested as an internal protective mechanism against apoptotic cell death, where Bcl-2 is persistently expressed in neurons that survive in ischemia. In the present study, brain regions that were selectively vulnerable to neurologic injury, particularly the neocortex and hippocampus, showed higher levels of Bcl-2 expression after HCA at 18°C compared with other brain regions (thalamus, cerebellum, and medulla). Moreover, profound hypothermia at 10°C resulted in a significant decrease in TUNEL staining in these brain regions. Although a concomitant increase in Bcl-2 expression was observed in the neocortex, it remains unclear whether profound hypothermia deters from neuronal injury by activation of anti-apoptotic protein Bcl-2 expression (Ananiadou et al, 2007). (Table 3)

<table>
<thead>
<tr>
<th>Sensory Cortex</th>
<th>18°C</th>
<th>10°C</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL</td>
<td>3.88±0.13**</td>
<td>1.60±0.31***</td>
<td>0.14±0.14</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>0.83±0.31</td>
<td>1.8±0.31*</td>
<td>1.8±0.63</td>
</tr>
</tbody>
</table>

Table 3. TUNEL Scores and Bcl-2 Immunoreactivity in the Sensory Neocortex of Animals Treated with HCA at 18°C or 10°C Compared to Controls

All values are expressed as mean ± SE.
*p<0.05 compared to values from animals treated with 18°C HCA.
**p<0.0001 compared to values from animals treated with 18°C HCA.
***p<0.002 compared to normal control levels
8.5 Morphological and ultrastructural evidence of neural protection during profound cooling

Necrosis can be characterized by passive cell swelling, rapid energy loss, and generalized disruption of internal homeostasis with lysis of the nucleus, intranuclear organelles and plasma membranes leading to the release of intracellular components that induce a local inflammatory response that in turn, result in edema and injury to neighboring cells. Morphologically, cell death is characterized by swelling of organelles and rupture. Necrotic cell death is characterised by inflammation and wide-spread damage. In contrast to those observed in cell necrosis, the morphological changes that occur during apoptotic cell death include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Kerr et al., 1972). In continuation of the above studies, we assessed the morphological evidence that profound cooling of the cortex to 10°C can reduce neurological injury during hypothermic circulatory arrest (HCA) in our porcine model. Electron microscopy assessed ultrastructural changes indicative of activation of programmed cell death.

Parafin embedded samples described above 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 one observer using a JEOL 100CX electron microscope who was instructed to find 10 representative neurons (per experimental brain specimen) as identified by a typical nucleus and surrounding perikaryon. Two blinded investigators using an objective grading system analyzed electron micrographs of these neurons. Each investigator was asked to examine each neuron for evidence of nuclear changes (chromatin dispersion or clumping), for the presence of cytoplasmic changes, for the overall shape of the neuron (shrunken, 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 compared with matched controls; each finding was indicated as mild, moderate, or severe depending on its frequency.

Electron microscopic observations in our study provided no appreciable morphological evidence to confirm apoptosis or necrosis of the sensory cortical neurons in this acute paradigm of HCA. In general, neurons showed normal nuclear and cytoplasmic morphology in all three treatment groups, with only minor ultrastructural changes observed after HCA at 18°C. There was no evidence of cells swelling. The neurons of the sensory cortex in control animals had large round or oval nuclei with an evenly distributed chromatin. No discontinuities were found in cytoplasmic membrane and nucleolmma. Well-developed rough endoplasmic reticulum (RER) that was arranged in parallel stacks was observed in the cytoplasm. Polyribosomes formed characteristic rosettes, and mitochondria appeared normal in all control animals. Blood capillaries were surrounded by...
thin astrocytic end feet and showed endothelial cells with euchromatic nuclei, tight junctions between the adjacent plasma membranes.

Compared to normal controls, treatment with HCA at 18°C (group A) resulted in minor ultrastructural alterations in the sensory cortex. (Table 4) Most neurons exhibited a pale round or oval nuclei. Nucleoli were intact, although sometimes they were localized in eccentric positions. Plasma and nuclear membranes remained intact, as did the mitochondria, which maintained their normal appearance, with recognizable cristae. While most neurons had slightly dilated RER and Golgi apparatus, some cells displayed more significant edema and morphological modifications of their organelles and dilated mitochondria. (Figure 3). In some cases, polysomes were disassociated, displaying desegregated ribosomes. In addition, some neurons, also exhibited some chromatin clumps. Although some mitochondria were slightly dilated, they showed an otherwise normal morphology.

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**Table 4. Ultrastructural Changes in the Sensory Neocortex of Animals Treated with HCA at 18°C or 10°C Compared to Controls**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall shape of the neuron</th>
<th>Nuclear changes</th>
<th>Cell organelles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shrunken 10°C 18°C</td>
<td>Swollen 10°C 18°C</td>
<td>Chromatin Dispersion 10°C 18°C</td>
</tr>
<tr>
<td>1</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>2</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>3</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>4</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
</tr>
</tbody>
</table>

- No changes noted compared to control
+ Positive observation compared to control animals
Deep hypothermia at 10°C resulted in negligible ultrastructural changes in the sensory cortex. Most neurons exhibited pale round or oval nuclei and intact nucleoli. In a few cases, the nucleoli were localized in eccentric positions. Plasma and nuclear membranes remained intact, and the structure of the cytoplasmic organelles was similar to that observed in control animals.

The first stage or the decision phase of apoptosis is initiated after an appropriate stimulus. This is referred to as the genetic control point of cell death and appears to be regulated by the Bcl-2 family of genes. The “execution phase” which follows is responsible for the morphologic changes of apoptosis (Kam and Ferch, 2000). The absence of clear morphologic evidence of apoptosis potentially suggests that these observations may represent an early point of activation of the apoptotic pathway (decision phase), which is supported by the Bcl-2 expression findings. As important regulators of the “decision phase,” an increase of Bcl-2 may represent an internal protective mechanism against apoptotic cell death. Bcl-2 is persistently expressed in neurons that survive in ischemia. The sensory cortex is selectively vulnerable to neurologic injury and showed high levels of Bcl-2 expression after HCA at 18°C compared (Ananiadou et al., 2008). Moreover, profound hypothermia at 10°C resulted in a significant decrease in TUNEL(+) staining in this brain region. The concomitant increase in Bcl-2 expression that was observed in the sensory neocortex, suggests that profound hypothermia (at 10°C) may deter from neuronal injury by activation of anti-apoptotic protein Bcl-2 expression (Almeida et al., 2000). Although subtle, electron microscopy showed that at 18°C cells exhibited dilation of the rough endoplasmic reticulum and mitochondria, ribosome detachment and Golgi derived vacuolation, while at 10°C cells exhibited only dilation of the rough endoplasmic reticulum and ribosome detachment, indicating Phase II and Phase I of the apoptotic process, respectively.

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 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 detachment of ribosomes, along with Golgi derived vacuolation. According to Portera-Cailliau and colleagues (Portera-Cailliau, et al., 1997), these findings suggest that the sensory cortex was in Phase II of the apoptotic process. At 10°C hypothermia, the ultrastructural findings indicate that the sensory cortex was in Phase I, showing only 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.

Although subtle, electron microscopy showed that at 18°C cells exhibited dilation of the rough endoplasmic reticulum and mitochondria, ribosome detachment and Golgi derived vacuolation, while at 10°C cells exhibited only slight changes. Our findings of significantly reduced TUNEL(+) staining, a concomitant increase in Bcl-2 expression and slightly decreased ultrastructural evidence of activation of programmed cell death support that deep hypothermia at 10°C further protects the sensory neocortex.

9. Conclusions

Cardiac surgeons are faced with the challenge of protecting the brain during the sensitive time of interruption of normal cerebral blood flow. The brain is an exceptionally complex
organ with a functional anatomy that is difficult both to understand and assess. Experimental and clinical studies have shown that the mechanism of neural injury is multifactorial. As such, discussions regarding the best surgical strategies for neuroprotection during circulatory arrest are formidable, at best. Although we are armed with excellent experimental and clinical studies that demonstrate the deleterious effects of prolonged exposure to cardiopulmonary bypass (CPB) on brain function and structure, the various neuroprotective strategies, particularly that of deep hypothermic circulatory arrest (DHCA) remain an issue of debate. This is related in part to the gap between the basic science understanding of brain injury caused by these events and the clinical application of various neuroprotective strategies and their subsequent clinical outcomes.

Our goal has been to assess a possible mechanism of the neuronal injury (eg apoptosis) following DHCA. As this appears to involve a subtle and complex cascade of events, we decided to apply a paradigm that on the one hand may not be totally clinically relevant, but on the other hand would allow a robust response for assessment. Further study is clearly warranted to unravel relevant mechanisms and sensitive markers, which in turn, would allow us to appreciate the potential clinical relevance of these experimental findings. Evaluating various strategies and treatments in animal studies in order to determine clinical feasibility remains a challenge. Animal models have contributed immensely to our understanding of cerebral consequences of HCA, with several animal models having been used. To date, the preclinical investigation of cerebral injury mechanisms related to deep hypothermic circulatory arrest has been limited to large-animal models (porcine, canine and ovine). These models are expensive, personnel demanding, cumbersome and are usually performed without validated neuropsychologic assessment. Rodent models have been attempted to overcome some of these disadvantages, although treatment effects cannot always be confirmed in the rat model. Ultimately, however, each experimental model system from cell cultures to rats, to large animals and ultimately to clinical trials, have their advantages and disadvantages, and ultimately their place in these investigations.

There is now convincing evidence that there is a general relationship between CNS damage and increasing duration of DHCA. Although one of the goals of experimental studies is to assess the upper safe limit of DHCA, in order to do so we must more clearly understand the mechanism of cerebral injury. In most animal models, an extended period of arrest is necessary to produce a consistent and reproducible level of neuronal injury that would facilitate elucidating the mechanisms of injury, as well as the efficacy of potential neuroprotective interventions. Many large animal models require DHCA for at least 90-120 minutes in order to demonstrate neurologic deficits. Although such prolonged DHCA interval might not be considered clinically realistic, they may be more appropriate for demonstrating the molecular pathways behind acute neuronal injury and hence, modes of intervention.

Profound hypothermia of the brain results in a reduction of cerebral blood flow and steady state cerebral oxygen consumption (considered a true index of brain metabolic activity). Research in laboratory animals and clinical observations have now documented that considerable residual cerebral metabolism remains with cooling to levels of 15-18°C, particularly when cooling interval are short. Both experimental and clinical paradigms are faced with unresolved issues, including cooling gradients, nonuniformity of brain cooling, rewarming, pH management, among others.

Various strategies have been addressed in an effort to reduce neurological complications, including profound hypothermia, antegrade cerebral perfusion, retrograde cerebral
perfusion, etc, each with their advantages and disadvantages. Cold reperfusion has shown promising results in animal studies and needs further clinical evaluation, while pharmacological interventions may offer a very promising pathway for preventing cerebral injury.

Experimental study of a neuroprotective strategy includes appropriate selection of an animal model, as well as functional indices. Of the available animal models, selection is made with respect to their relevance and feasibility of assessing the parameters of interest. The later are identified in the context of the available data. Investigations of promising neuroprotective methods require validation in an experimental model (validation study), use of the method in experimental settings to optimize cerebral protection during CPB and DHCA (performance study) and test its utility during routine cardiac surgery (clinical study). Despite the plethora of experimental and clinical studies, we still require a clearer understanding of the pathophysiologic consequences of HCA. This information will be pivotal in clinical decision-making, including when to initiate circulatory arrest and the appropriate interval.

Delayed cell death via apoptotic pathways is of special interest because of the potential for intervention. Although apoptosis is believed to play a part in the cerebral injury, its role has generally been identified through histologic techniques in animal models. These snapshots do not permit a clear delineation of the time-line of apoptosis in the course of HCA. Because it clinical role is not clear, therapies have yet to be designed for the specific purpose of inhibiting apoptosis. Both the cascade of events and identification of pharmacologic agents that can act on molecular mediators require active investigation.

Rewarming represents a critical time period, during which any additional harm to cerebral cells might induce permanent injury or even precipitate their death. How rapidly a stable energetic and biochemical homeostasis can be obtained in order to prevent the occurrence of secondary injuries remains unclear.

Optimal perfusion characteristics required to reduce neurologic morbidity remain important issues for experimental study. While there is ample evidence supporting the effectiveness of antegrade perfusion, its optimal delivery and perfusion characteristics remain unclear. Overall, there are still many gaps in our knowledge about how to best study cerebral outcome following DHCA. The wealth of available evidence suggests that investigations require coordinated efforts by multiple research groups, pursuing systematic, multilevel research – spanning from cell cultures, to various animal model systems ranging from rodents to large animals and ultimately to clinical trials.

10. Acknowledgements

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11. References


Front Lines of Thoracic Surgery collects up-to-date contributions on some of the most debated topics in today's clinical practice of cardiac, aortic, and general thoracic surgery, and anesthesia as viewed by authors personally involved in their evolution. The strong and genuine enthusiasm of the authors was clearly perceptible in all their contributions and I’m sure that will further stimulate the reader to understand their messages. Moreover, the strict adhesion of the authors’ original observations and findings to the evidence base proves that facts are the best guarantee of scientific value. This is not a standard textbook where the whole discipline is organically presented, but authors’ contributions are simply listed in their pertaining subclasses of Thoracic Surgery. I’m sure that this original and very promising editorial format which has and free availability at its core further increases this book’s value and it will be of interest to healthcare professionals and scientists dedicated to this field.

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