Apoptotic Neuronal Death following Deep Hypothermic Circulatory Arrest in Piglets

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Background: Deep hypothermic circulatory arrest (DHCA), as used in infant heart surgery, carries a risk of brain injury. In a piglet DHCA model, neocortical neurons appear to undergo apoptotic death. Caspases, cytochrome c, tumor necrosis factor (TNF), and Fas play a role in apoptosis in many ischemic models. This study examined the expression of these factors in a DHCA piglet model.

Methods: Thirty-nine anesthetized piglets were studied. After cardiopulmonary bypass (CPB) cooling of the brain temperature to 19°C, DHCA was induced for 90 min, followed by CPB rewarming. After separation from CPB, piglets were killed at 1, 4, 8, 24, and 72 h and 1 week. Caspase-8 and -3 activity, and concentrations of TNF-α, Fas, Fas-ligand, cytochrome c, and adenosine triphosphate (ATP) were measured in the neocortex by enzymatic assay and Western blot analysis. Caspase-8 and -3 activity and cell death were examined histologically. Significance was set at P < 0.05.

Results: In neocortex, damaged neurons were not observed in control (no CPB), rarely observed in CPB (no DHCA), and rarely observed in the DHCA 1-h, 4-h, and 1-week reperfusion groups. However, they were seen frequently in the DHCA 8-, 24-, and 72-h reperfusion groups. Although neuronal death was widespread 8–72 h after DHCA, cortical ATP concentrations remained unchanged from control. Piglets caspase-3 and -8 activities were significantly increased at 8 h after DHCA, and caspase-3 concentration remained elevated for as long as 72 h. Caspase-3 and -8 activity was also observed in damaged neocortical neurons. Cytosolic cytochrome c and Fas were significantly expressed at 1 and 4 h after DHCA, respectively. Fas-ligand and TNF-α were not observed in any group.

Conclusion: After DHCA, induction of apoptosis in the neocortex occurs within a few hours of reperfusion and continues for several days. Increased Fas, cytochrome c, and caspase concentrations, coupled with normal brain ATP concentrations and apoptotic histologic appearance, are consistent with the occurrence of apoptotic cell death.

AS SURVIVAL for neonates with congenital heart disease has improved, concern has shifted to the neurologic morbidity afflicting 5–40% of survivors. Neurologic morbidity manifests as seizures, diminished cognition, language delays, altered tone, impaired coordination, and/or attention deficit hyperactivity disorder. Global cerebral ischemia related to cardiopulmonary bypass (CPB) and deep hypothermic circulatory arrest (DHCA), used in the repair of the cardiovascular defects, appears to play an important role in the neurologic morbidity.

After a global ischemic event, cells in certain brain regions are known to die, whereas other cells in the same region or different regions do not. This phenomenon, referred to as selective vulnerability, occurs in mature and immature brain. In neonates, neurons and oligodendrocytes in the neocortex and hippocampus are selectively vulnerable to death after DHCA. Many of these cells die by a process called apoptosis.

In apoptosis, cell death occurs despite adequate cellular energetics and results from the activation of specific genes, receptors, and enzymes, through which cells break up into membrane-bound bits for engulfment by resident macrophages. Cell death by necrosis, in contrast, involves energy failure, catalysis, and membrane rupture, which elicits inflammation and secondary injury. Central to the execution of apoptosis are caspases, a family of cysteine proteases, of which caspase-8 and -3 are particularly important.

Two principal pathways exist for the activation of caspase-3. In the extrinsic pathway, classic inflammatory factors such as Fas and tumor necrosis factor (TNF-α) can activate caspase-8 through cell surface receptors. Caspase-8 in turn activates caspase-3, which destroys cytoskeletal proteins and DNA. In the intrinsic pathway, caspase-3 is activated by the release of cytochrome c from dysfunctional mitochondria.

Previous findings with a newborn pig DHCA model suggest that many neurons in the neocortex die by apoptosis, beginning within hours of reperfusion and continuing for several days postoperatively. Evidence of apoptosis includes characteristic changes in cell structure and in situ DNA fragmentation. These findings, however, are not specific for apoptosis.

This article is accompanied by an Editorial View. Please see:
In the present study we sought to characterize the activity of key apoptotic proteins and enzymes, such as caspase-3 and -8, in relation to neocortex neuronal death after DHCA in newborn pigs.

METHODS

Surgical Preparation

After approval by the Institutional Animal Care and Use Committee of the Joseph Stokes Jr. Research Institute, 39 piglets aged 5–10 days were studied. Anesthesia was induced with intramuscular ketamine (33 mg/kg) and acepromazine (3.3 mg/kg), followed by tracheal intubation, mechanical ventilation, and intravenous catheter insertion. Anesthesia was maintained with intravenous fentanyl (initial loading dose of 25 μg/kg, then 10 μg·kg⁻¹·h⁻¹) and droperidol (0.25 mg·kg⁻¹·h⁻¹).

A femoral arterial catheter was inserted to monitor arterial pressure (P23XL Transducer; Spectramed, Critical Care Division, Oxnard, CA), glucose concentrations (Surestep; Lifescan, Milpitas, MN), blood gases, pH, and hemoglobin concentrations (iSTAT; iSTAT Company, East Windsor, NJ). Expired carbon dioxide was measured (Normocap; Daytex-Ohmeda Division, Instrumentarium Corporation, Helsinki, Finland) and electrocardiographic monitoring (ECG; Hewlett-Packard, Federal Republic of Germany) was performed. Thermistors (Yellow Springs Instruments, Yellow Springs, OH) were inserted into the cranial epidural space, rectum, and esophagus to monitor brain and core body temperatures.

Protocol

Animals were divided into eight groups. Controls (group 1) underwent surgical preparation and were killed (n = 8). Group 2 underwent surgical preparation and then hypothermic CPB, followed by 1-day survival (n = 3). Groups 3–8 underwent surgical preparation and then hypothermic CPB and DHCA, followed by 1-h (n = 5), 4-h (n = 6), 8-h (n = 5), 24-h (n = 5), 72-h (n = 4), or 1-week (n = 3) survival. In groups 2–8, physiologic variables were recorded after surgical preparation (before CPB), after CPB cooling, during CPB rewarming, and 2 h after discontinuation of CPB. In group 1, physiologic variables were recorded after surgical preparation.

Brain Tissue

In survival groups, intramuscular ketamine and acepromazine were administered before killing. Then the scalp was reflected, after which the skull and dura over the left cerebral hemisphere were removed. Intravenous heparin (300 U/kg) was administered. Frontal, parietal, and occipital neocortical tissue samples (200–300 mg) were obtained with a biopsy drill, which aspiration the samples into a liquid nitrogen-cooled reservoir. The frozen tissue was stored in liquid nitrogen until analysis.

After pentobarbital (100 mg/kg) was administered intravenously (euthanasia), chilled saline 0.9% (1 l) and then 4% paraformaldehyde in 0.1 M phosphate-buffered saline (1 l, pH 7.4) were infused into the carotid artery to fix the brain in situ. After the brain was removed in toto, the right hemibrain was isolated and cut coronally into 2.5-mm blocks. Alternating blocks were prepared for frozen or paraffin-embedded sections. The blocks destined for frozen section were stored at 4°C in 25% sucrose, followed 2 days later by immersion in cold isopentane before storage at −70°C.

The blocks destined for paraffin sectioning were dehydrated in ethanol and xylene (Citadel 2000; Shandon-Lipshaw, Pittsburgh, PA) and embedded in paraffin (Histocryl 1160; Leica, Deerfield, IL). One 8-μm section was cut (Microtome 2155; Leica) from a paraffin block and stained with hematoxylin and eosin to determine cell damage. One 40-μm section was cut from a frozen block (Jung Biocut BSF-5TC; Leica) with use of tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) to determine immunohistochemically the location of caspase-3 and -8 activities.

Neurologic Examination

In the 24-h, 72-h, and 1-week survival groups, a neurologic examination was performed before killing. Points were assessed for deficits in level of consciousness (range, 0–25), respiration (0–5), cranial nerve function (0–6), sensory function (0–14), gait (0–25), and behavior (0–20). The scores from each category were summed. The minimum score (0) represents no deficits (normal examination findings), whereas the maximum score (95) indicates severe impairment (brain death).

Cardiopulmonary Bypass

Through a right-neck incision, the carotid artery and external jugular vein were exposed, through which cannulae (Bio-Medicus; Medtronic, Minneapolis, MN) were advanced to the ascending aorta and right atrium for CPB. Heparin (200 U/kg) was administered intravenously. After arterial cannulation, 10 ml/kg blood was collected for transfusion after CPB.

The CPB circuit used a bubble oxygenator (Bio-2; Baxter Healthcare Corporation, Santa Ana, CA) with an arterial filter (LPE 1440 extracorporeal filter; KOL Bio-Medical Instruments, Chantilly, VA) receiving oxygen at a rate of 1 l/min and a nonpulsatile roller pump (RS 7800; Renal Systems, Minneapolis, MN) flowing at 100 ml·kg⁻¹·h⁻¹. Arterial blood gas management followed a-stat principles. The CPB prime contained pig whole blood, heparin (2,000 U), fentanyl (50 μg), pancuronium (1 mg), calcium chloride (500 mg), dexamethasone (30 mg/kg), cefazolin (25 mg/kg), furosemide (1 mg/kg), and sodium bicarbonate (25 mEq). Plasma-lyte A (Baxter, Deerfield, IL) was added to yield a hematocrit of 25% during CPB.
Hypothermia was induced with CPB and ice bags around the head and body. At a brain temperature of 19°C, rewarming was initiated in the CPB group, whereas DHCA was induced for 90 min in the DHCA groups. To rewarm, CPB pump flow and arterial perfusate were increased gradually to 100 ml \( \cdot \) kg\(^{-1} \) \( \cdot \) h\(^{-1}\) and 38°C, respectively, facilitated with a radiant-heating lamp and circulating-water blanket. After 10 min of CPB, the heart was defibrillated as necessary, and mechanical ventilation was resumed.

At a brain temperature of 28°C, mannitol (0.5 g/kg) was administered. CPB was discontinued when all body temperatures were greater than 32°C (approximately 40 min of CPB rewarming).

After CPB, cannulae were removed, protamine (4 mg/kg) was administered intravenously, and the neck incision was sutured closed. The blood collected before CPB was transfused over 1-2 h to maintain a mean arterial pressure >50 mm Hg, after which dextrose (5%) in lactated Ringer’s solution was infused intravenously (4 ml \( \cdot \) kg\(^{-1} \) \( \cdot \) h\(^{-1}\)). In the 1-, 4-, and 8-h DHCA reperfusion groups, anesthesia was continued until killing. In the 24-h, 72-h, and 1-week survival groups, anesthesia was discontinued, and the trachea was extubated when purposeful movements, airway reflexes, strength, and regular breathing had returned.

**Histologic Outcome**

Hematoxylin and eosin-stained slides containing frontoparietal neocortex, corresponding to the areas that were biopsied in the opposite hemisphere, were evaluated for neuronal cell death, inflammation, hemorrhage, and infarction. The slides were scored semiquantitatively on a scale of 0–5: 0 = no damage (normal neuronal structure); 1 = rare damage (<1% neurons dead; no inflammation or infarction); 2 = mild (1–5% neurons dead; no inflammation or infarction); 3 = moderate (6–15% neurons dead; no inflammation or infarction); 4 = severe (16–30% neurons dead, inflammation, or infarction [one or all of these]); and 5 = very severe (>30% of all neurons dead; inflammation and infarction).\(^6\) Apoptotic cells were defined by the presence of nuclear karyorrhexis and minimal cytoplasmic change, whereas necrotic cells were identified by a pyknotic nucleus or no nucleus, along with a swollen, eosinophilic cytoplasm.

**Biochemistry**

The adenosine triphosphate (ATP) concentrations were measured by the Luciferin-Luciferase reaction. In brief, 1 mg frozen neocortical tissue was dissolved in 100 \( \mu \)l 0.1-N NaOH in methanol and brought to 0.3 ml with \( \text{H}_2\text{O}\). After heating (90°C) for 15 min, 5 \( \mu \)l was added to 100 \( \mu \)l Luciferin-Luciferase solution (Sigma Chemical, St. Louis, MO). Standard ATP concentrations (Sigma Chemical Co) were also prepared. The sample and standard reactions were read in a Luminometer (LKB 1250; Perkin Elmer-Wallac, Turku, Finland).

An ApoAlert Caspase Colorimetric Assay Kit (Clontech, Palo Alto, CA) was used to measure caspase-3 and -8 activities. Frozen neocortical samples (5–10 mg) were homogenized in 0.5 ml cell lysis buffer (4°C) containing 25 mM HEPES (pH, 7.5), 5 mM EDTA, 5 mM MgCl\(_2\), 5 mM dithiothreitol (DTT), and 10 mM/ml each of pepstatin, leupeptin, aprotinin, and PMSF. Homogenates were centrifuged at 10,000 rpm for 10 min. The supernatant was brought to 50 \( \mu \)l with cell lysis buffer, to which was added 50 \( \mu \)l of 2X reaction buffer, followed by 5 \( \mu \)l p-nitroanalide (pNA) substrate. For caspase-3 activity the pNA substrate was DEVD-pNA (1 mM), and for caspase-8 activity it was Ac-IETD-pNA (4 mM). The 1-ml 2X reaction buffer was prepared by adding 5 \( \mu \)l of 1.0 mM DTT to 1 ml reaction buffer. Standard pNA concentrations were prepared in lysis buffer. The caspase-3 and caspase-8 reactions were incubated at 37°C for 1 and 2 h, respectively. Sample and standard solutions were measured at 405 nm (DU-640 spectrophotometer; Beckman, Fullerton, CA). Supernatant total protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA).

**Western Blot Analysis**

The same supernatant was used for Western blot analysis as for biochemical assays. Separate gels were run for each DHCA reperfusion group, containing representative samples in eight wells and molecular standards in two wells. Because of sample concentration and volume considerations, the lanes for the CPB, DHCA 4-h reperfusion, DHCA 8-h reperfusion, DHCA 24-h reperfusion, and DHCA 1-week reperfusion groups were loaded with 0.25 \( \mu \)g protein, and the lanes for the control, DHCA 1-h reperfusion, and DHCA 72-h reperfusion groups were loaded with 0.50 \( \mu \)g protein. These gels were the source of blots against antibodies for caspase-3 and -8, TNF-\( \alpha \), Fas, and Fas-ligand. For cytochrome c, the samples were concentrated (Speedvac Concentrator; Savant Instruments, Farmingdale, NY) and the lanes were loaded with 5 \( \mu \)g protein from each DHCA group.

Protein samples were mixed with Laemmli sample buffer consisting of 1% SDS, 0.1% 2-mercaptoethanol, and 0.1% bromophenol blue in a ratio of 1:1 (vol/vol). After boiling for 3 min, the proteins were separated by 8–16% gradient sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad). After electrophoresis (200 V) at room temperature for 30 min, proteins were transferred onto a nitrocellulose membrane (50 V) and stored overnight at 4°C. The blots were then immersed in 2% bovine serum albumin for 30 min at room temperature on a rocker platform. The primary antibody (10 \( \mu \)l) was added and allowed to react overnight at 4°C. The blot was then washed in TBST (50 mM Tris [pH, 7.5], 150 mM NaCl, and 0.05% Tween-20), after

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were initially treated with 1% H2O2 for 30 min to inactivate endogenous peroxidase and then 3 ml of 2% BSA containing the secondary antibody. The blot was wrapped in cellophane and rinsed in water. The blot was exposed to secondary antibody (1:500 dilution) for 1 h. Afterward, 75 μl diaminobenzidine solution (Santa Cruz) was added for 5–10 min until color was apparent.

Table 1. Physiologic Data in Cardiopulmonary Bypass Group and Deep Hypothermic Circulatory Arrest Group

<table>
<thead>
<tr>
<th></th>
<th>Pre-CPB</th>
<th>CPB cool</th>
<th>CPB rewarm</th>
<th>Post-CPB</th>
</tr>
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<td>pH</td>
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<td>7.41 ± 0.10</td>
<td>7.41 ± 0.16</td>
<td>7.43 ± 0.07</td>
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<tr>
<td>Pco2, mmHg</td>
<td>37 ± 6</td>
<td>39 ± 7</td>
<td>37 ± 13</td>
<td>38 ± 6</td>
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<td>Po2, mmHg</td>
<td>427 ± 129</td>
<td>680 ± 159</td>
<td>415 ± 48</td>
<td>400 ± 155</td>
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<tr>
<td>Hematocrit, %</td>
<td>25 ± 3</td>
<td>24 ± 2</td>
<td>25 ± 3</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>59 ± 10</td>
<td>37 ± 10</td>
<td>51 ± 12</td>
<td>90 ± 17</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>168 ± 40</td>
<td>245 ± 42</td>
<td>239 ± 53</td>
<td>181 ± 46</td>
</tr>
<tr>
<td>Tbrain °C</td>
<td>37 ± 1</td>
<td>19 ± 1</td>
<td>33 ± 2</td>
<td>37 ± 1</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SD.
CPB = cardiopulmonary bypass; MAP = mean arterial pressure; Pco2 = arterial blood partial pressure of CO2; Po2 = arterial blood partial pressure of O2; Tbrain = brain temperature.

which it was immersed in 2% BSA containing the secondary antibody.

After washing with TBST, the blot was immersed in LumiGLO (Cell Signaling, Beverly, MA) for 3 min and rinsed in water. The blot was wrapped in cellophane and exposed to CL-exposure film (Pierce, Rockford, IL) for 15 sec and subsequently developed.

Immunohistochemistry
Free-floating, 40-μm frozen sections were immersed in 50 mM Tris (pH, 7.5) with 0.1% Triton X-100. Sections were initially treated with 1% H2O2 for 30 min to inactivate endogenous peroxidase and then 3 ml of 2% bovine serum albumin for 30 min. After the addition of primary antibody to caspase-3 or caspase-8 (Cell Signaling) at 4 °C overnight, sections were washed with 3 ml 50 mM Tris (pH, 7.5) with 0.1% Triton X-100, followed by exposure to secondary antibody (1:500 dilution) for 1 h. Three-milliliter avidin–biotin peroxidase complex (Santa Cruz) was then added for 1 h. Afterward, 75 μl diaminobenzidine solution (Santa Cruz) was added for 5–10 min until color was apparent.

Tissues were then washed with water to stop the reaction. They were brushed on poly-L-Lysine-coated slides and left to dry at room temperature. Tissue sections were immersed in a 1:10 dilution of hematoxylin for 30 sec as a counterstain and dehydrated with increasing concentrations of ethanol and then xylene for 5 min.

Statistical Analysis
Data are presented as mean ± SD. Groups were compared by analysis of variance (ANOVA). For significant F values, multiple means were compared with Tukey test. A P value of less than 0.05 was considered statistically significant.

Results
Table 1 displays the average and SD physiologic data for all groups combined during the study. There were no

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Fig. 1. Neurologic performance scores on postoperative day (POD) 1 (n = 12), POD 2 (n = 7), POD 3 (n = 7), and POD 7 (n = 3) after deep hypothermic circulatory arrest (DHCA) and for cardiopulmonary bypass (CPB)–only animals (n = 3). Scoring system ranged from 0 to 95, corresponding to no neurologic deficits (normal examination findings) to severe damage (brain death), respectively. Box shows 25–75 interquartile range; bar shows median score. Performance score increased significantly at POD 1 and 2, indicating impaired neurologic function (P < 0.05).
significant differences in physiologic data between the groups. CPB cooling, rewarming, and total CPB durations were 28 ± 9 min, 41 ± 10 min, and 70 ± 17 min, respectively. Neurologic impairment was apparent at 1 and 2 days postoperatively after DHCA (fig. 1). Signs of neurologic impairment manifested mainly as gait disturbance and diminished alertness. Neurologic impairment was not observed in CPB-only groups.

In neocortex, damaged neurons were not observed in the control group, rarely in the CPB and DHCA 1-h, 4-h, and 1-week reperfusion groups, and often in the DHCA 8-h, 1-day, and 3-day reperfusion groups (fig. 2). Although neuronal death was widespread at 8–72 h after DHCA, cortical tissue ATP concentrations remained similar to those in the control group (table 2). Although not quantitated, damaged neurons were usually apoptotic in appearance (fragmented, rounded, dense chromatin with minimal cytoplasmic change) and less often necrotic (nuclear pyknosis with a swollen, eosinophilic cytoplasm) (fig. 3). Damaged neurons usually appeared in clusters located in the superficial (layers 2 and 3) neocortex.

Caspase-3 activity was significantly increased 8 h to 3 days after DHCA, in comparison with that in the control and CPB groups (fig. 4). Caspase-3 active fragments were present on Western blots as early as 4 h after DHCA and as long as 3 days after DHCA (fig. 5) and were not present in control, CPB, or other DHCA groups. Curiously, caspase-3 fragments were biphasic, as the fragments were not observed at 8 h after DHCA. Caspase-8 activity was significantly increased only at 8 h after DHCA. On Western blots, caspase-8 active fragments were present at 8 h after DHCA, as well as 1 day after DHCA.
DHCA and 1 day after CPB only (Fig. 5). Caspase-3 and -8 activity appeared in neurons located mainly in superficial neocortex, although activity was also present in glial cells in cortical white matter and basal ganglia (Figs. 6 and 7).

Cytochrome c increased significantly 1 h after DHCA (Fig. 8). Fas increased significantly 4 h after DHCA; there was also a tendency for increased Fas (P = 0.07) at 1 h after DHCA (Fig. 9). Fas-ligand was not increased significantly (Fig. 9). On Western blots, TNF-α was not detected in the control, CPB, or DHCA groups.

**Discussion**

This study reveals that DHCA neuronal death involves activation of the apoptotic cascade. Cell death occurred primarily in the superficial neocortex with morphologic features consistent with apoptosis. Despite neuronal death, cortical tissue ATP concentrations were preserved. According to assay and Western blot results, caspase-3 activity coincided with cell death at 8–72 h after DHCA. Caspase-8 activation occurred at 8 h, Fas expression at 4 h, and cytochrome c release at 1 h after DHCA, showing initiation of the apoptotic cascade well before cell death was apparent. Apoptotic-appearing neurons showed caspase-3 and -8 active fragments in the neocortex. These enzyme activities and cell death were not observed in control and CPB groups. Our findings support the implication that caspase inhibitors and other suppressors of apoptosis might afford neuroprotection for DHCA in neonatal heart surgery.

Our model simulates neonatal heart surgery with CPB and DHCA. CPB is used to induce hypothermia and support circulation, as well as effect resuscitation and rewarming after DHCA. Although hypothermia confers neurologic protection, this protection is incomplete. After DHCA, neurons and oligodendrocytes are selectively vulnerable to die in the neocortex and less so in the hippocampus, striatum, and cerebellum. Selective vulnerability is not a result of uneven brain cooling, as brain temperature gradients are not observed during DHCA in our model. Previous work has shown that cell death occurs as early as 6 h after DHCA, peaking at 24–72 h, whereas neurologic function improves despite ongoing cell death.

The difference in time course between histology and function may result from the cells that are injured but not dead interfering with neurologic function, as opposed to dead cells, which can be compensated for by the other live cells. Neurologic deficits include gait disturbance and diminished alertness, consistent with the location of cell death in the neocortex. Cell death in this model appears to be apoptotic from morphological analysis.

The current study showed morphologic, histologic, and neurologic outcomes similar to those in prior investigations but yielded biochemical and molecular evidence of apoptosis. Specifically, the apoptotic cascade is initiated as soon as 1 h after reperfusion, well before cell death appears, and continues for 72 h after DHCA. Cell
death and the apoptotic cascade both diminished by 1 week after DHCA.

Certain regions of the brain have been shown to be selectively vulnerable to injury.3-6 These regions appear to differ between neonates and adults, as well as between hypothermic and normothermic ischemia. Bottiger et al.3 studied neuronal death after normothermic global ischemia in adult rats and found neurons in the hippocampus, cerebellum, striatum, thalamus, amygdala, and neocortex to be vulnerable. Laptook et al.4 studied global ischemia in normothermic and hypothermic piglets. They found that neurons in the neocortex, thalamus, basal ganglia, cerebellum, and brain stem were vulnerable after normothermic ischemia and that hypothermia does not confer neuroprotection equally to all brain regions.

In a DHCA puppy model, Mujscie et al.5 observed that the neocortex, basal ganglia, and amygdala were selectively vulnerable and that hypothermic protection was not equal in all regions. In our DHCA model, selective vulnerability was observed in similar locations in so far as the neocortex, hippocampus, striatum, and cerebellum were concerned.6 Our findings differed, however, in the amygdala, thalamus, and brain stem. These differences may represent differences in animal species and models of ischemia.

Evidence is accumulating for apoptosis in neuronal death following global ischemic insult.7-8 In apoptosis, cell death results from a regulated process involving the activation of specific genes, receptors, and proteins that ultimately cause DNA fragmentation and the destruction of cell structure. The apoptotic cascade depends on the activation of caspases, a family of cysteine proteases specific for substrates at the aspartate residue. Caspase-3 is known as the executioner caspase, because it directly activates a nuclease (ICAD) that cleaves DNA strands and destroys the cell cytoskeleton.

Two principal pathways for the activation of caspase-3 include Fas/FADD/caspase-8 (the extrinsic pathway) and cytochrome c/Apaf-1/caspase-9 (the intrinsic pathway). However, these signaling pathways interact with each other to amplify caspase-3 activity and apoptotic cell death.

In the extrinsic pathway, TNF-α and Fas-ligand are known to bind to cell surface receptors, which recruit the adaptor molecule Fas-associated death domain (FADD), and the zymogen form of caspase-8. After recruitment, procaspase-8 is proteolytically cleaved to gen-
erate two distinct subunits: a large (p18) fragment and a small (p10) fragment. These subunits assemble into a heterotetramer to activate caspase-3 by cleaving it into 17–21-kDa and 10–13-kDa subunits. Our enzymatic assays measured caspase-8 and caspase-3 cleavage activity, and Western blot analysis detected the active fragments of caspase-3 and -8.

In the intrinsic pathway, the mitochondrion increases membrane permeability and releases cytochrome c into the cytosol in response to ischemia. Cytochrome c forms a complex with Apaf-1, which in turn activates caspase-9 to activate caspase-3. Our Western blot analyses detected cytochrome c in cytosolic fractions of brain tissue homogenates.

In normothermic neonatal hypoxia-ischemia models, evidence of apoptosis includes the presence of damaged neurons with apoptotic structure and TUNEL positivity, as well as caspase activity and the expression of proapoptotic and antiapoptotic factors, such as bcl-2, bcl-x, and bax. Even more convincing evidence of apoptosis is the effectiveness of caspase inhibitors in affording neuroprotection after ischemia. During normal brain development, neurons in the neocortex are known to selectively die by apoptosis. Comparison of adult and neonatal models of hypoxia-ischemia suggests that apoptosis may be more prevalent in the immature brain.

Our study gives biochemical and molecular evidence of apoptosis in another neonatal hypoxia-ischemia model. Because enzymatic assays are not entirely specific for caspase-3 or caspase-8 enzymatic function, we used immunoblot analyses to confirm the presence of active fragments specific for each caspase. Our results implicated both the extrinsic and intrinsic mechanisms of apoptosis, as both caspase-8 activation and cytochrome c release were observed. The trigger for caspase-8 appeared to be Fas and not TNF-α. However, further experiments are necessary to functionally tie Fas to caspase-8 induction and exclude insensitivity of the blot for TNF-α. To confirm that caspase activity was located in the cells that were damaged, we observed active caspase-3 and -8 fragments in neurons in the superficial neocortex.

In our study, there were some discrepancies between the findings of the enzymatic assay and Western analysis. For caspase-8, enzyme activity was increased at 8 h reperfusion, whereas immunoblot activity was increased 8–24 h after reperfusion. For caspase-3, assays showed activity increased from 8 to 72 h after reperfusion, whereas blots showed biphasic active fragments with increases at 4 h and 24–72 h. The discrepancy between immunoblot and enzyme assay findings may arise from the lack of complete specificity of the enzyme assay and/or from differences in assay methods. The biphasic expression of caspase-3 fragments suggests the possibility of two pathways for activation, perhaps the intrinsic
and extrinsic pathways. Cytochrome \(c\), in the intrinsic pathway, was released at 1 h, which might account for the early caspase-3 increase, whereas caspase-8, in the extrinsic pathway, was active later, which might have played a role in the late caspase-3 increase.

It is also important to note the lack of significant expression of Fas-ligand, despite the clear presence of Fas active fragments at 4 h after DHCA. Because Fas-ligand is subject to rapid proteolytic cleavage, its expression is difficult to detect, our finding may reflect differences in the stability of Fas and Fas-ligand.

In summary, following DHCA, induction of apoptosis in the neocortex occurs within a few hours of reperfusion and continues for several days. Increased Fas, cytochrome \(c\), and caspase concentrations, coupled with normal brain ATP concentrations and apoptotic histologic appearance, are consistent with the occurrence of apoptotic cell death.

References