REGIONAL PATTERNS OF NEURONAL DEATH AFTER DEEP HYPOTHERMIC CIRCULATORY ARREST IN NEWBORN PIGS

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Objectives: Deep hypothermic circulatory arrest (DHCA) widely used during neonatal heart surgery, carries a risk of brain damage. In adult normothermic ischemia, brain cells in certain regions die, some by necrosis and others by apoptosis (programmed cell death). This study characterized regional brain cell death after DHCA in newborn pigs. **Methods:** Eighteen piglets underwent 90 minutes of DHCA and survived 6 hours, 2 days, or 1 week. Six piglets underwent surgery alone or deep hypothermic cardiopulmonary bypass and survived 2 days. Three piglets received no intervention (control). Brain injury was assessed by neurologic and histologic examination and correlated with perioperative factors. Apoptosis and necrosis were identified by light microscopic analysis of cell structure and in situ DNA fragmentation (TUNEL). **Results:** All groups subjected to DHCA had brain injury by neurologic and histologic examination, whereas the other groups did not. DHCA damaged neurons in the neocortex and hippocampus and occasionally in the striatum and cerebellum. Damaged neurons in the neocortex were mainly apoptotic and in the hippocampus, a mixture of necrotic and apoptotic neurons. Apoptosis and necrosis were apparent in all DHCA groups even though neurologic deficits improved over the week's survival. Neocortical and hippocampal damage correlated with blood glucose, hematocrit, and arterial PO₂ during and after cardiopulmonary bypass. **Conclusions:** In neonates, neocortical and hippocampal neurons are selectively vulnerable to death after DHCA. Both apoptosis and necrosis contribute to neuronal death, beginning early in reperfusion and continuing for days. These data suggest the need for several neuroprotective strategies tailored to the region and death process, initiated during the operation and continued after the operation. (J Thorac Cardiovasc Surg 1999;118:1068-77)

Deep hypothermic circulatory arrest (DHCA) is frequently used for repair of complex cardiovascular defects in neonates. Although this technique has contributed to successful heart operations in neonates, it

carries a risk of neurologic sequelae. These include seizures, motor dysfunction, cerebral palsy, developmental delay, and cognitive impairment in 5% to 45% of survivors.^{1,2} Neurologic sequelae appear to result from global ischemia (circulatory arrest), with cardiopulmonary bypass (CPB), deep hypothermia, hemodilution, postoperative care, and pre-existing disease as contributing factors.

After a global ischemic insult, certain neuronal subpopulations are known to die whereas others do not.^{3,4} This phenomenon, referred to as selective vulnerability, occurs in adult and neonatal brain. Neurons in the hippocampus, cerebellum, striatum, amygdala, lateral thalamic nucleus, and third to fifth layers of the neocortex are selectively vulnerable to ischemia in adults. Loss of neurons in these regions is responsible for the impairment of memory, cognition, emotional state, and motor

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function seen in adults after cardiac arrest. Selective vulnerability appears to differ between adults and neonates and between normothermic and hypothermic ischemia.³⁻⁸

Recent work indicates that some selectively vulnerable neurons in adults and neonates die after ischemia by a process called *apoptosis* (programmed cell death).^{3,9} In apoptosis, cell death is orchestrated, involving the activation of specific genes and enzymes, through which cells neatly commit suicide, breaking up into membrane-packaged bits for removal by resident macrophages.^{10,11} Cell death by necrosis, on the other hand, is uncontrolled, involving energy failure, catalysis, and membrane rupture, spilling cellular contents to elicit inflammation and secondary injury. Apoptosis occurs during normal brain development and helps shape brain architecture through the death of specific cell populations during fetal and early postnatal life.^{12,13} Apoptosis also plays a role in neuronal cell death after hypoxia-ischemia, brain trauma, and neurodegenerative diseases, although its role relative to necrosis remains unsettled.^{3,9-11} In neonates, apoptosis may be favored over necrosis as a cell death process after hypoxia-ischemia.⁹ Whether apoptosis plays a role in neuronal cell death after DHCA in neonates is unknown.

In the present study, we sought to characterize brain injury after DHCA in a newborn pig model to determine the selectively vulnerable cell populations and the contribution of apoptosis to cell death.

Methods

Surgical preparation. We studied 28 piglets aged 3 to 10 days (1.7-2.9 kg). The Institutional Animal Care and Use Committee of the Joseph Stokes Research Institute approved the studies. After anesthetic induction with intramuscular ketamine (33 mg/kg) and acepromazine (3.3 mg/kg) and tracheal intubation, catheters were inserted into an extremity vein and femoral artery. Anesthesia was maintained with inhaled halothane (0.5%) and intravenous fentanyl (25 µg/kg). Cephazolin (25 mg/kg) was administered intravenously. Arterial pressure, end-expired carbon dioxide, electrocardiogram, blood gases, pH, hemoglobin, and glucose concentrations were monitored. Thermistors (models 555 and 401, Yellow Springs Instrument Company) were inserted into the cranial epidural space, rectum, and esophagus to monitor brain and core temperatures.

Through an incision in the right side of the neck, the carotid artery and external jugular vein were exposed. After intravenous heparin (200 units/kg) was administered, cannulas (Medtronic Bio-Medicus) were advanced to the aorta and right atrium for CPB. The CPB circuit used a bubble oxygenator (Bio-2, Baxter Cardiology), a 40-µm arterial filter (Kol Bio-Medical Instruments), and a nonpulsatile roller pump (RS 7800, Renal Systems) flowing at 100 mL · kg⁻¹ ·

min. The pump prime contained pig whole blood, heparin 2000 units, fentanyl 50 µg, pancuronium 2 mg, calcium chloride 500 mg, dexamethasone 30 mg, cephazolin 25 mg/kg, and sodium bicarbonate 25 mEq. Electrolyte solution (Plasma-Lyte A, Travenol Laboratory) was added to yield a hematocrit value of 20% to 25% during CPB. Blood gases were managed by alpha-stat principles.

During CPB cooling, the perfusate was kept 5°C to 10°C less than body temperature. At 19°C (brain), DHCA lasting 90 minutes was induced and confirmed by asystole and no arterial pressure. Ice bags were positioned around the head to maintain the brain temperature at 19°C. During CPB reperfusion, arterial perfusate was kept 5°C to 10°C greater than body temperatures, the maximum being 38°C. After 15 minutes' reperfusion, the heart was defibrillated. When all temperatures were more than 33°C, CPB was stopped, cannulas were removed, protamine 4 mg/kg was injected intravenously, and incisions were closed.

Postoperatively, inspired oxygen concentration and minute ventilation were adjusted to maintain arterial PCO₂ at 35 to 45 mm Hg and arterial PO₂ greater than 75 mm Hg. Dextrose 5% in lactated Ringer solution was infused intravenously (4 mL · kg⁻¹ · h⁻¹). When purposeful movements, airway reflexes, and regular breathing had returned, the trachea was extubated. The animals were inspected frequently after DHCA. If they were unable to feed from the trough, bottle feeding or intravenous fluids were initiated.

Experimental protocol. Piglets were randomly assigned to control, surgery, CPB, and DHCA groups. The control group (n = 3) received no intervention (healthy animals put to death for histologic analysis). The surgical group (n = 3) received the surgical preparation without CPB. The CPB group underwent deep hypothermic CPB (n = 3) without DHCA (cooling to deep hypothermia followed by rewarming). Surgery and CPB groups survived 2 days. The DHCA groups survived either 6 hours (n = 6), 2 days (n = 6), or 1 week (n = 6). Physiologic data were recorded before CPB, during deep hypothermic CPB (before DHCA), during warm CPB (after DHCA), and 15 minutes and 2 hours after ending CPB.

Neurologic outcome. A neurologic performance score was performed in the 2-day and 1-week survivor groups.¹⁴ The score consists of a physical examination with points given for specific deficits in level of consciousness (range 0-25), cranial nerve function (range 0-6), sensory function (range 0-14), gait (range 0-25), and behavior (range 0-20). The score is weighted between categories to reflect the components of the neurologic examination and degree of functional disability associated with that category.¹⁴ The scores from each category were summed. The minimum score, 0, represents no deficits (normal examination results), whereas the maximum score, 95, indicates severe damage.

Brain histology. After survival for the designated intervals, piglets were re-anesthetized, given anticoagulants (intravenous heparin 300 units/kg), and put to death (intravenous pentobarbital 100 mg/kg). Chilled saline solution 0.9% (1 L) followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline solution (1 L, pH 7.4) were infused

Table I. Physiologic data before, during, and after CPB

	<i>Before</i>	<i>cCPB</i>	<i>wCPB</i>	<i>After</i>
pH	7.43 ± 0.08	7.46 ± 0.10	7.41 ± 0.11	7.40 ± 0.07
PCO ₂ (mm Hg)	35 ± 8	34 ± 10	25 ± 10*	36 ± 7
PO ₂ (mm Hg)	437 ± 159	664 ± 107	537 ± 140	228 ± 167
MAP (mm Hg)	60 ± 10	45 ± 15*	51 ± 11†	85 ± 17*
Hct (%)	24 ± 4	25 ± 2	25 ± 2	31 ± 3*
T _{brain} (°C)	36 ± 1	19 ± 0.6*	32 ± 1*	36 ± 1
Glucose (mg/dL)	144 ± 26	236 ± 51*	218 ± 24*	235 ± 19*

Mean ± SD, n = 21. *Pco₂*, Arterial partial pressure of carbon dioxide; *Po₂*, arterial partial pressure of oxygen; *MAP*, mean arterial pressure; *Hct*, hematocrit value. Arterial blood gases were measured at 37°C. *cCPB*, Deep hypothermic CPB before circulatory arrest; *wCPB*, warm CPB after circulatory arrest.

*P < .001 versus baseline by repeated-measures ANOVA.

†*P* = .01 versus baseline by repeated-measures ANOVA.

into the aortic root to fix the brain in situ. The brain was removed in toto, immersed in 4% paraformaldehyde, and stored at 4°C in phosphate-buffered saline solution. After a superficial cut was made along the undersurface of the right hemi-brain to identify it from the left, the whole brain was cut coronally into 5-mm blocks. The tissue blocks were dehydrated in ethanol and xylene (Citadel 2000, Shandon-Lipshaw) and embedded in paraffin (Histoembedder 1160, Leica). Two 8-μm sections cut from each tissue block (Microtome 2155, Leica) were mounted onto slides. One section was stained with hematoxylin and eosin to characterize cell damage; the other section was prepared for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) to detect in situ DNA fragmentation. The TUNEL assay was performed as described.¹⁵ Each assay included positive and negative control slides (mouse breast tissue (Oncor) and brain tissue without the terminal deoxynucleotidyl transferase, respectively).

Histologic outcome. All slides were scored by a neuropathologist blinded to the experimental group. Brain regions evaluated included the neocortex, hippocampus, striatum (caudate-putamen), thalamus, amygdala, cerebellum, and brain stem (pons and medulla), chosen for their known vulnerability to hypoxia-ischemia. Cell damage was categorized as either necrotic or apoptotic according to classic morphologic criteria.¹⁶ Although these criteria are not absolute indicators of the cell death process, they serve as a useful first step to elucidate it. Further, these categories are not always mutually exclusive and may not represent the only possible modes of cell death. Apoptotic cells were defined by the presence of nuclear karyorrhexis (fragmented, rounded, dense chromatin) and minimal cytoplasmic change, whereas necrotic cells were identified by a pyknotic nucleus or no nucleus (ghost neuron) along with a swollen, eosinophilic cytoplasm. In addition to nuclear and cytoplasmic changes, sections were evaluated for inflammation, hemorrhage, and infarction. TUNEL(+) cells were identified by a red-stained, condensed nucleus with apoptotic bodies, along with a diminutive or absent cytoplasm.

To describe the extent of damage and apoptosis among the brain regions, we used a semiquantitative score. On each slide, the regions were scored on a scale of 0 to 4. Hematoxylin-

eosin slides were scored as follows: 0 = normal neuronal structure; 1 = rare clusters (<5) of damaged neurons; 2 = occasional clusters (5-15) of damaged neurons; 3 = frequent clusters (>15) of damaged neurons; and 4 = diffusely distributed damaged neurons. The corresponding scores for the TUNEL slides were as follows: 0 = no TUNEL(+) apoptotic cells; 1 = rare clusters of TUNEL(+) apoptotic cells; 2 = occasional clusters of TUNEL(+) apoptotic cells; 3 = frequent clusters of TUNEL(+) apoptotic cells; and 4 = diffusely distributed TUNEL(+) apoptotic cells. Histopathologic and TUNEL scores were the average among of the slides: neocortex (7 slides), hippocampus (2 slides), striatum (2 slides), thalamus (2 slides), amygdala (1 slide), cerebellum (2 slides), and brain stem (2 slides).

To assess the density of damaged and apoptotic neurons in a cluster, we estimated the percentage of damaged and TUNEL(+) cells in afflicted areas of the neocortex and hippocampus. Four 2.5-mm² areas containing damaged or TUNEL(+) neurons were selected in each region. The number of damaged, TUNEL(+), and normal-appearing neurons were counted in the neocortex (from the surface of the gray matter down to the border with the white matter) and in the hippocampus (along the CA1 sector).

Statistical analysis. Data are presented as mean ± SD. Comparisons among groups were made by analysis of variance (ANOVA) for continuous variables or Kruskal-Wallis ANOVA for discontinuous variables. When a significant overall *F* was found, pairwise multiple comparisons were made with the use of the Tukey or Mann-Whitney test. Pearson correlation coefficients were calculated between neurologic outcome variables (eg, histopathologic score) and perioperative variables (eg, arterial pressure). Multivariable regression was explored between neurologic outcome variables and perioperative variables having correlation coefficients, with .01 level of significance to adjust for the multiple correlation tests.

Results

Twenty-seven piglets survived according to protocol; one piglet died, cause unknown, the first night after DHCA. Table I displays perioperative factors before,

during, and after CPB in the survivors of the DHCA and CPB groups. These groups did not differ significantly ($P > .2$). The duration of CPB cooling, CPB reperfusion, and total CPB was 23 ± 5 minutes, 39 ± 5 minutes, and 62 ± 9 minutes, respectively. DHCA and CPB groups were extubated, respectively, 4.9 ± 1 hours and 2.9 ± 0.5 hours after ending CPB ($P = .01$).

All piglets in the DHCA 2-day and 1-week survival groups displayed neurologic deficits. Deficits included disturbed gait (all piglets), feeding difficulty (75% of piglets), abnormal tone (50% of piglets), and impaired consciousness (15% of piglets). The number of piglets with deficits and the severity of those deficits improved significantly with survival time (Fig 1). Deficits were present in all piglets (100%) on postoperative day 1, 10 of 12 piglets (83%) on postoperative day 2, and 1 of 6 piglets (16%) on postoperative day 7 ($P < .001$). Neurologic deficits were not observed in the control, surgical, or CPB groups.

All animals in the DHCA groups had histologic evidence of brain damage. In the neocortex, damaged neurons appeared in clusters, distributed symmetrically between the hemi-brains (Fig 2, A and B). Outside of the clusters, the neuropile was normal. In a cluster, damaged neurons appeared apoptotic and were located predominantly in superficial gray matter (layers 2 and 3), interspersed among normal cells. Mitotic figures were occasionally seen near the apoptotic clusters. In the neocortex, necrosis was rarely seen: only one or two small areas of selective neuronal necrosis or infarction were seen, respectively, in 33% and 11% of piglets. Except for the rare area of infarction, nonneuronal cells were not damaged. In the hippocampus, damage was variable among animals, located in CA1-3 (77% of piglets) and less often in CA4 (22% of piglets) and fascia dentata (38% of piglets). In CA1-4, damage was a mixture of necrotic and apoptotic neurons (Fig 2, C). In the fascia dentata, damaged neurons were mainly apoptotic.

Table II displays the hematoxylin-eosin histologic scores in control, surgical, CPB, and DHCA groups. In the DHCA groups, injury occurred in the neocortex (100% of piglets), hippocampus (88% of piglets), striatum (22% of piglets), and cerebellum (22% of piglets). Injury was apparent by 6 hours of reperfusion and was significantly worse in the neocortex and hippocampus than in the striatum and cerebellum. No animals in the control, surgical, or CPB groups had brain damage. Histopathologic score for the neocortex correlated significantly with neurologic performance score on postoperative day 2 ($r = 0.67$, $P = .02$), whereas it did not for the hippocampus ($r = 0.39$, $P > .2$) or for either region on postoperative day 1 ($r = 0.41$ - 0.46 , $P > .2$).

Fig 1. Neurologic performance scores after DHCA in piglets surviving 2 days ($n = 6$) and 1 week ($n = 6$). Scores 0 and 95 represent no neurologic deficits (normal examination) and severe damage (brain death), respectively. Bar shows mean score. Performance score decreased significantly with survival time, indicating improved neurologic function.

All animals in the DHCA groups had TUNEL(+) cells with apoptotic structure. In neocortex, TUNEL(+) cells usually appeared in clusters (Fig 3, A), similar in distribution to the clusters of hematoxylin-eosin apoptotic neurons (Fig 2, A). Outside of the clusters, TUNEL(+) cells were rarely seen. Within a cluster, TUNEL(+) cells were located mainly in superficial gray matter, interspersed among normal neurons. In the hippocampus, TUNEL(+) cells were also seen (Fig 3, B).

Table III displays the TUNEL (+) scores in the groups. In control, surgery, and CPB groups, TUNEL(+) cells were observed occasionally in all brain regions as isolated cells (never in clusters). After DHCA, TUNEL positivity increased significantly in the neocortex at 6 hours', 2 days', and 1 week's survival and in the hippocampus at 6 hours' survival. TUNEL positivity decreased significantly from 6 hours to 1 week after DHCA. In the other regions, TUNEL positivity after DHCA remained similar ($P > .2$) to control, surgery, or CPB groups.

Fig 4 illustrates the cell counts for hematoxylin-eosin-damaged and TUNEL(+) neurons in the neocortex and hippocampus. In control, surgery, and CPB groups, no damaged neurons were observed and TUNEL(+) cells were infrequent ($<0.3\%$ of neurons). After DHCA, both damaged and TUNEL(+) cells increased significantly in the neocortex ($P = .002$, $P < .001$) and in the hippocampus ($P = .02$, $P < .001$). At 6 hours' and 2 days' survival, damaged neurons were sig-

Fig 2. Brain damage after DHCA in a representative 2-day survival piglet. **A**, Schematic illustration of a coronal section showing the distribution of clusters of damaged neurons, marked by the dots. Cut at bottom denotes right hemi-brain; dashed lines the separation of gray and white matter; lateral ventricles at midline. Schematic created from a 1 $\times$ projection of a hematoxylin-eosin slide, followed by a high-power (100 $\times$) scan to identify the clusters. **B**, Photomicrograph of a cluster of damaged neurons in the superficial neocortex. Damaged neurons appeared apoptotic (arrowheads). Mitotic figures (arrow) often appeared near the clusters (original magnification $\times 400$). **C**, Photomicrograph of hippocampus CA1 showing mixture of necrotic (arrowheads) and apoptotic (arrow) neurons (original magnification $\times 400$).

Table II. Histopathologic scores following surgery, deep hypothermic CPB, and DHCA

	Neocortex	Striatum	Thalamus	Hippocampus	Amygdala	Cerebellum	Brain stem
Control	0	0	0	0	0	0	0
Surgery	0	0	0	0	0	0	0
CPB	0	0	0	0	0	0	0
DHCA, 6 hours	1.7 ± 0.5*†	0.1 ± 0.2	0	1.5 ± 0.8*†	0	0.1 ± 0.2	0
DHCA, 2 days	2.11 ± 0.7*†	0.2 ± 0.2	0	2.3 ± 1*†	0	0.1 ± 0.2	0
DHCA, 1 week	1.9 ± 1.2*†	0.5 ± 1.2	0	2 ± 1.3*†	0	0.6 ± 1	0

Mean ± SD, n = 27. Score range 0-4, representing no damage (0) and extensive damage (4). Surgery group received surgery for CPB but did not experience CPB. CPB group received surgery and deep hypothermic CPB but no circulatory arrest. DHCA groups received surgery, deep hypothermic CPB, and circulatory arrest followed by 6 hours', 2 days', or 1 week's survival. Surgery and CPB groups survived 2 days.

*P < .001 versus surgery, CPB, and control by ANOVA.

†*P* < .001 versus striatum, thalamus, amygdala, cerebellum, and brain stem by repeated-measures ANOVA.

Table III. TUNEL(+) histologic scores after surgery, deep hypothermic CPB, and DHCA

	Neocortex	Striatum	Thalamus	Hippocampus	Amygdala	Cerebellum	Brain stem
Control	0.6 ± 0.3§	0.3 ± 0.3	0.2 ± 0.3	0.2 ± 0.2	0.3 ± 0.5	0.1 ± 0.2	0.1 ± 0.2
Surgery	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.3	0.5 ± 0.5	0.6 ± 0.5	0.3 ± 0.3	0.3 ± 0.3
CPB	0.8 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.2
DHCA, 6 hours	2.4 ± 0.3*†‡	1.6 ± 1.3	1.1 ± 0.7	1.1 ± 0.4*	1.3 ± 0.8	0.2 ± 0.4	0.5 ± 0.5
DHCA, 2 days	1.9 ± 0.5*†‡	0.6 ± 0.6	0.6 ± 0.5	0.5 ± 1	0.5 ± 0.5	0.6 ± 0.8	0.3 ± 0.5
DHCA, 1 week	1.6 ± 0.6*†‡	0.6 ± 0.4	0.5 ± 0.4	0.4 ± 0.5	0.2 ± 0.4	0.2 ± 0.2	0.3 ± 0.5

Mean ± SD, n = 27. Score range 0-4, representing no TUNEL(+) neurons (0) and extensive TUNEL(+) neurons (4). Groups as in Table II.

*P < 0.001 versus control, surgery, and CPB groups.

†*P* = .005 versus DHCA 1 week.

‡*P* < .001 versus other regions.

§*P* = .02 versus other regions.

Table IV. Perioperative factors correlated with neurologic outcome

Outcome	Physiologic factor	<i>r</i>	<i>P</i> value
Performance score day 2	Duration CPB cooling	0.66	.01
Neocortex			
% Cells damaged	Glucose, wCPB	0.53	.02
	PO ₂ , 2 hours after	-0.67	.002
Histopathology score	Hct, 2 hours after	-0.47	.04
Hct, wCPB		-0.47	
% Cells TUNEL(+)	MAP, wCPB	-0.53	.02
Hippocampus			
Histopathology score	Glucose, cCPB	-0.60	.008
	Glucose, wCPB	-0.64	.004
% Cells TUNEL(+)	Glucose, 30 min after	-0.51	.03
TUNEL(+) score	PO ₂ , wCPB	-0.49	.03
	Hct, wCPB	-0.48	.04

Abbreviations as in Table I. n = 18, except for performance score (n = 12). After, After discontinuing CPB after DHCA.

nificantly greater than TUNEL(+) cells in the hippocampus, whereas they were similar (*P* > .2) in the neocortex. At 1 week's survival, damaged neurons were greater than TUNEL(+) cells in both the hippocampus and neocortex. The percentage of damaged neurons in the neocortex correlated significantly with neurologic performance score on postoperative day 2 (*r* = 0.72, *P* = .01), whereas it did not in the hip-

pocampus or in either region on postoperative day 1 (*r* = 0.43-0.52, *P* > .2).

Several perioperative factors emerged as significant correlates with neurologic outcome (Table IV). Poor neurologic performance on postoperative day 2 correlated with increased CPB cooling duration. Neocortical damage correlated with increased serum glucose during CPB rewarming, and decreased arterial PO₂ and

Fig 3. Apoptosis in the brain after DHCA in a 6-hour survival piglet. **A**, Cluster of TUNEL(+) apoptotic neurons (nucleus red stained) interspersed among normal neurons (nucleus blue stained) in the superficial neocortex (original magnification $\times 400$). Inset at higher magnification ($\times 600$) shows apoptotic bodies in the nucleus (dark red dots). **B**, TUNEL(+) apoptotic neurons in the CA1 hippocampus (original magnification $\times 600$).

hematocrit value during CPB rewarming and after. Hippocampal damage correlated with decreased serum glucose during CPB. Increased TUNEL positivity in the hippocampus and neocortex correlated with decreased mean arterial pressure, arterial PO_2 , and hematocrit value during CPB rewarming. No multivariable regression models were found to significantly predict outcome.

Discussion

The current study characterized brain injury after DHCA in a newborn animal model in terms of cell type, region, and process. We found neurons in the neocortex and hippocampus to be vulnerable to death after

DHCA. Neurons in the striatum and cerebellum were also vulnerable, although much less so, and nonneuronal cells and neurons in other regions were resistant. In the neocortex, apoptosis was the predominant death process, whereas in the hippocampus it was a mixture of necrosis and apoptosis. Cell death occurred within 6 hours of reperfusion and continued for 1 week, even though neurologic deficits improved significantly during this time. These findings indicate that DHCA brain damage is not a uniform process and that it occurs over several days, suggesting the need for several neuroprotective strategies, initiated intraoperatively and continued postoperatively.

In animal models of global ischemia, age and tem-

Fig 4. Percent of neurons in the neocortex or hippocampus (CA1) showing damage or apoptosis (TUNEL+). C/S/CPB represents the average of control, surgery, and CPB groups. DHCA-6h, DHCA-2d, and DHCA-1wk indicate DHCA groups surviving 6 hours, 2 days, or 1 week. Damaged and apoptotic neurons in the neocortex and hippocampus increased significantly ($P < .001$) in all DHCA groups compared with C/S/CPB. In the neocortex, the increase in damaged and apoptotic neurons was similar, whereas in the hippocampus, apoptotic neurons were significantly less than damaged neurons. * $P = .04$ damaged versus TUNEL+; # $P = .02$ damaged 1-week versus 6-hour and $P = .15$ damaged versus TUNEL+. Line and bar = mean $\pm$ SD.

perature have been shown to influence which cell populations die.^{3-8,17} Vulnerable cell populations in adult normothermic ischemia include neurons in the hippocampus, cerebellum, striatum, thalamus, amygdala, and neocortex (third to fifth layers).^{3,6} Glia, endothelium, and smooth muscle are relatively resistant to death, as are neurons in other regions. By comparison, neurons in the striatum and neocortex (second and third layers) were vulnerable in newborn normothermic ischemia.⁴ Hypothermia does not appear to confer protection equally to all neuronal populations. For example, after hypothermic global ischemia, thalamus, hippocampus, and striatum continued to be damaged even though the other vulnerable regions were protected.^{4,17} Although the mechanism of selective vulnerability is not fully understood, the metabolic makeup, trophic factor availability, and synaptic connectivity of the cells in the region appear to play a role.

During normal brain development, neurons in certain regions (eg, the neocortex) are also known to selectively die by a process called apoptosis.^{12,13} Apoptosis uses endogenous suicide programs. Certain factors (eg, cytokines, glutamate) activate these programs, which result in a characteristic cell degeneration, in which the cell breaks up into membrane-bound bits, which are engulfed by resident macrophages.^{10,11} Consequently, apoptosis lacks the inflammation and secondary tissue damage of necrosis. Several of the pro-apoptotic and

anti-apoptotic factors also play a role in cellular proliferation.^{10,12} It is not uncommon to see cell division and apoptosis simultaneously in the same region.

Apoptosis has also been found to play a role in neuronal death after ischemia and other neurologic diseases.^{3,9-11,15,18,19} Evidence for apoptosis in ischemia includes damaged neurons with apoptotic structure and TUNEL positivity, as well as caspase activity and pro-apoptotic and anti-apoptotic factor expression in the damaged region. More convincing is the neuroprotection afforded by caspase inhibitors or in animals genetically deficient in caspases.^{9,18,19}

Previous work described apoptosis in adult and neonatal models of global ischemia.^{3,9} In these models, apoptotic neuronal death began within hours of reperfusion and continued for several days. The contribution of apoptosis to ischemic cell death varied with brain region and severity of the insult, being prominent in the neocortex and hippocampus after mild to moderate ischemia.^{3,10} In our study, apoptosis displayed a time course and regionality similar to these models, suggesting that anti-apoptotic drugs should be examined for neuroprotection in relation to DHCA.

Morphologic and biochemical criteria distinguish apoptosis from necrosis.^{10,16} A hallmark of apoptosis is DNA fragmentation into ordered oligonucleosomes with 3'-OH end groups, detectable with in situ labeling (eg, TUNEL). This method, however, is not complete-

ly sensitive or specific, as it is possible to see apoptotic cells without TUNEL labeling and necrotic cells with TUNEL labeling.^{10,16,20} Moreover, apoptosis and necrosis are not always mutually exclusive processes in ischemia; both may be active in a dying cell.^{10,16,20} In our study, the combination of morphology, TUNEL labeling, mitotic figures, and lack of inflammation indicates apoptosis as a process by which many neurons in the neocortex and hippocampus die after DHCA.

The temporal pattern of brain damage and apoptosis after DHCA merits comment. We observed histologic cell death, TUNEL labeling, and apoptosis early (6 hours) in both the neocortex and hippocampus. This rapid cell death is consistent with cell suicide programs, which can kill a cell in 2 to 3 hours.^{12,13} Although TUNEL labeling was maximal early, we also observed TUNEL labeling and apoptotic cell death for days after DHCA. This may indicate the continued presence of programmed cell death activators, some programs working slowly, or attempts at repairing injured neurons that were later aborted (then activating apoptosis). We also noted more apoptosis in the neocortex than hippocampus, perhaps indicating different suicide programs or activators to the programs in these regions.

The perioperative factors associated with the DHCA damage provide clues to the mechanism of the injury. Increased arterial pressure, PO_2 , and hematocrit value during CPB rewarming and after CPB were associated with improved neurologic outcome, suggesting that local tissue hypoxia during reperfusion may contribute to damage. The role of glucose was conflicting between the neocortex and hippocampus, as increased glucose worsened damage to the former and ameliorated it to the latter. The effect of glucose on ischemic brain damage in neonates is conflicting,²¹⁻²³ in contrast to adults, in whom glucose consistently aggravates ischemic brain damage.²⁴ Brain temperature heterogeneity did not contribute to brain injury as brain temperature gradients are less than 1°C during DHCA in our model.²⁵

Our results differ from previous work in developing animal models of DHCA.^{7,8} The predominant lesion in the 1-month-old pig was selective neuronal necrosis and infarction in the cerebellum, striatum, and neocortex.⁸ This difference from our study may reflect brain maturity responses to DHCA between 1-month-old and newborn pigs. The main lesion in the newborn dog was selective neuronal necrosis in the deep neocortex and striatum.⁷ This difference from our study may reflect the DHCA models, as the newborn dog study did not use CPB. DHCA without CPB might contain a compo-

nent of incomplete ischemia during reperfusion, damaging the watershed regions (eg, deep neocortex).

Our model differs from DHCA used in clinical practice. The advantages of our closed-chest CPB model for research include no bleeding, pulmonary dysfunction, or physical disability from surgery, simplifying postoperative care and neurologic assessment. The bubble oxygenator requires less prime volume, given the requirement of blood prime in the newborn pig. The advantage of 90 minutes' DHCA for research is the consistency of brain damage, minimizing the number of study animals. The distribution of apoptosis and necrosis with varying DHCA durations warrants study, as DHCA in clinical practice is usually briefer. Our model's disadvantage is the possibility of carotid artery ligation and gaseous emboli contributing to the brain damage. Several observations argue against this possibility. First, ligation of one carotid artery in piglets has no effect on cerebral blood flow during normal or hypoxic-ischemic conditions.²⁶ Second, the ischemic lesions were not consistent with unilateral carotid artery ligation or emboli because of the symmetry of injury between the hemi-brains, selectivity of cells injured, and lack of injury in the CPB group.

Despite improved surgical results in neonates over the past decade, neurologic sequelae continue to occur.^{1,2} Most neuroprotective strategies have been directed intraoperatively to prevent necrotic cell death. Our findings implicate another cell death pathway, apoptosis, suggesting another target for neuroprotective strategies.

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