Cardiothoracic Anesthesia, Respiration and Airway

Low hematocrit worsens cerebral injury after prolonged hypothermic circulatory arrest in rats

[Un niveau réduit d'hématocrite aggrave les lésions cérébrales après un arrêt circulatoire hypothermique prolongé chez le rat]

Su Dian-San PhD MD, Wang Xiang-Rui PhD MD, Zheng Yongjun PhD MD, Zhao Yan-Hua PhD MD

Purpose: This study tests the hypothesis that low hematocrit (Hct) worsens cerebral injury after prolonged hypothermic circulatory arrest (HCA) in rats, and the mechanism involves variable expression of the genes C-Fos, Bcl-2 and Bax.

Methods: A rat HCA model was developed, and 40 animals were randomly assigned to four groups: Sham (sham) group, or Hct groups of Hct 10%, Hct 20% and Hct 30%. After 90 min of HCA at 18°C, physiologic variables were recorded and brain morphological changes were evaluated with light and electron microscopy. Expressions of C-Fos, Bcl-2, Bax in various brain areas were measured by the reverse transcriptase polymerase chain reaction and standard immunohistochemistry techniques.

Results: The number of injured neurons in the hippocampus CA1 and parietal cortex in the Hct 10% group (CA1: 11.44 ± 2.52 ; cortex: 13.65 ± 2.31) exceeded the mean number of injured neurons in the Hct 20% group (CA1: 8.29 ± 1.31 ; cortex: 10.68 ± 1.24 ; $P < 0.05$) and the Hct 30% group. Mean mitochondrial injury scores were greatest at lower Hct levels, while the expression of C-Fos and Bax were highest in the Hct 10% group and lowest in the Hct30% group ($P < 0.05$). In contrast, the expression of the Bcl-2 mRNA was greatest in the Hct 30% group ($P < 0.05$).

Conclusion: Low Hct worsens cerebral injury after prolonged HCA and CPB in rats, which may relate in part to the variable expression of the genes C-Fos, Bcl-2 and Bax.

Objectif: Cette étude vérifie l'hypothèse qu'un niveau réduit d'hématocrite (Hct) aggrave les lésions cérébrales survenant après un arrêt circulatoire hypothermique (ACH) chez le rat et que le mécanisme implique l'expression variable des gènes C-Fos, Bcl-2 et Bax.

Méthode : Un modèle d'ACH a été développé chez le rat et on a réparti de façon aléatoire 40 animaux dans quatre groupes : un groupe sans intervention et des groupes Hct de 10 %, 20 % et 30 %. Après un ACH pendant 90 min à 18°C, on a mesuré les variables physiologiques et on a évalué les changements morphologiques dans le cerveau avec la microscopie optique et électronique. On a mesuré l'expression de C-Fos, Bcl-2 et Bax dans différentes régions du cerveau par la réaction en chaîne transcriptase inverse – polymérase et par des techniques habituelles d'immunohistochimie.

Résultats : Les neurones CA1 de l'hippocampe et du cortex pariétal ont été atteints en plus grand nombre dans le groupe Hct 10 % (CA1 : $11,44 \pm 2,52$; cortex : $13,65 \pm 2,31$) que dans le groupe Hct 20 % (CA1 : $8,29 \pm 1,31$; cortex : $10,68 \pm 1,24$; $P < 0,05$) et dans le groupe Hct 30 %. Le score moyen d'atteinte mitochondriale était plus élevé à des niveaux réduits d'Hct. C'est dans le groupe Hct 10 % que l'expression de C-Fos et de Bax était la plus élevée et dans le groupe Hct 30 % qu'elle était la plus faible ($P < 0,05$). Par contre, l'expression de l'ARN messenger Bcl-2 était plus élevée dans le groupe Hct 30 % ($P < 0,05$).

Conclusion : Les lésions cérébrales suite à un ACH et une circulation extra-corporelle prolongés sont aggravées par une réduction de l'Hct. Ceci peut être relié à l'expression variable des gènes C-Fos, Bcl-2 et Bax.

From the Department of Anesthesiology, RenJi Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China.

Address correspondence to: Dr. Wang Xiang-Rui, Department of Anesthesiology, RenJi Hospital, School of Medicine, Shanghai Jiaotong University, 1630 Dong Fang Road, Shanghai, post code 200127, China. Phone: +86 21 58752345, 3198; Fax: +86 21 50903239; E-mail: wangxr2010@yahoo.com

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HEMODILUTION has been widely applied for management of hypothermic cardiopulmonary bypass (CPB) for many years. It was first introduced to decrease homologous blood use,¹ thereby avoiding the “homologous blood syndrome”.² Later, the effects of hemodilution in counteracting the deleterious rheologic consequences of deep hypothermia, such as increased viscosity and red cell rigidity, came to be considered important in minimizing brain injury associated with deep hypothermic circulatory arrest (HCA).³

Although experience with cardiovascular surgery in adults refusing transfusion suggests that very low hematocrit (Hct) levels can be tolerated,⁴ the optimal Hct level for cerebral protection during CPB and HCA has not been defined. Published expert opinion⁵ has suggested that Hct levels of greater than 15% to 20% are safe during CPB in adults, but Duebener *et al.*⁶ recently demonstrated that perfusion of the cerebral microcirculation is not impaired by high Hct values (30%) during CPB compared with moderate (20%) or low (10%) Hct values. They also reported evidence of increasingly inadequate cerebral tissue oxygenation and greater white cell endothelial activation as Hct values decreased during CPB.⁶ The authors proposed that their results of increased ischemia and inflammatory tissue injury with greater hemodilution explained earlier findings of improved neurological outcomes associated with higher Hct.^{7,8} In a single-centre randomized trial with blinded assessments, Jonas *et al.*⁹ demonstrated that greater hemodilution (21%) during hypothermic CPB is associated with adverse perioperative and developmental outcomes in infants. Habib *et al.*¹⁰ also suggest from an observational study that increasing degrees of hemodilution during CPB are associated with greater perioperative vital organ dysfunction/morbidity as well as greater short- and intermediate-term mortality rates. Hematocrit values < 22% were found to be an independent predictor of operative mortality and morbidity.

Gaining insight into the mechanisms of dilutional anemia, and in particular the molecular pathways possibly involved, has been limited by the relative inability to study brain tissue after recovery from HCA and CPB. With the development of a rat model of CPB,¹¹ we have established a technique to sample brain tissue after HCA and CPB for molecular analysis, using well established molecular tools.

Apoptosis is a well documented series of events that results in the programmed self-destruction of cells. Its stimulus for initiation is variable but includes ischemia and other stresses.^{12,13} These stresses induce an

intracellular molecular cascade that ultimately results in self-destruction of tissue. Apoptosis is responsible for the long-term loss of neuronal tissue after cerebral ischemia,¹⁴ but its potential role in cerebral injury induced by HCA and CPB at different Hct levels has not been established. We therefore hypothesized that low Hct levels worsen cerebral injury after prolonged hypothermic circulatory arrest in rats, which possibly involves variable expression of the genes C-Fos, Bcl-2 and Bax.

Methods

This study was approved by the School of Medicine, Shanghai Jiaotong University Animal Care and Use Committee. All procedures were performed in accordance with the guidelines of the National Institutes of Health (NIH) for animal care (guide for the care and use of laboratory animals, Health and Human Services, NIH Publication No. 86-23, revised 1985).

Groups and conditions

Male Sprague-Dawley rats, aged 14 weeks or more, and each weighing 400–450 g were chosen for the study. Forty rats were randomly assigned in equal numbers into one of four groups. The three hemodilution groups had targeted Hct values of 10%, 20% and 30% (Groups: Hct 10%; Hct 20% and Hct 30%, respectively), while a fourth group served as the sham operation group. Anesthesia, ventilation, cannulation and heparinization were identical in all groups throughout the experiments, with the exception that rats in the sham operation group did not undergo hemodilution and CPB.

Priming of the hemodiluted groups

Target Hct were achieved by varying the composition of the CPB priming fluid. In the Hct 30% group, the CPB prime consisted of 10 mL heparinized donor blood. Blood was used from donor rats who were anesthetized with 10% chloral hydrate 2 mL·kg⁻¹, and blood was withdrawn through the femoral artery. The remainder of the prime consisted of Ringer's lactate 4 mL, mannitol (20%) 1 mL, heparin (100 UI) 1 mL and 6% hydroxyethyl starch 4 mL (HAES-sterile® Fresenius Kabi, Bad Homburg, Germany). In the Hct 20% group the CPB prime consisted of Ringer's lactate 10 mL, 20% mannitol 1 mL, heparin (100 UI) 1 mL and hydroxyethyl starch 4 mL. In the Hct 10% group the CPB prime solution was identical to that of the Hct 20% group, with the exception that before CPB, 10 mL of blood was withdrawn and replaced with Ringer's lactate 5 mL and 6% HAES 5 mL administered intravenously.

Cardiopulmonary bypass circuit preparation

Using a CPB model developed and described previously,¹⁴ the CPB circuit was prepared with a non-pulsatile roller pump (Masterflex®; Cole-Parmer Instrument Co., Vernon Hills, IL, USA) and sterile silicone tubing, with an inner diameter of 1.6 mm (Tygon®; Cole-Parmer Instrument Co., Vernon Hills, IL, USA). The circuit flow was set at a rate of 120 mL·kg⁻¹·min⁻¹. Venous return was drained by gravity into a 10-mL sterile reservoir consisting of a 10-mL syringe. The membrane oxygenator was designed specially for small animals, having a surface area of 0.1 m². A heat exchanger with a surface area of 0.03 m² was fixed to the oxygenator (Fudan Biomaterials Co. Ltd, Shanghai, China). The total prime volume of the circuit was 20 mL, of which 8 mL was in the oxygenator. Priming for each experiment was carried out according to group allocation.

Anesthesia and monitoring

The rats were anesthetized with 10% chloral hydrate (2 mL·kg⁻¹) and anesthesia was maintained with the same solution (0.8 mL·kg⁻¹·hr⁻¹). After endotracheal intubation, the animal's lungs were ventilated mechanically with 100% oxygen. The right femoral artery was cannulated with a 22G catheter to monitor mean arterial blood pressure and provide a sampling port for arterial blood gas analysis. Each rat received heparin 150 IU *iv* after placement of the first intravascular catheter. The right internal jugular vein was cannulated with a 22G catheter to monitor oxygen saturation jugular vein (SjvO₂). The electrocardiogram was monitored using three *sc* needle electrodes, and rectal temperature was recorded.

Cardiopulmonary bypass

A multi-orificed 14G *iv* catheter was inserted in the right common jugular vein and advanced 3.5 cm until its tip was placed near the junction of the superior vena cava and right atrium. This served as the venous inflow for the CPB circuit, drawing blood from the right atrium to the CPB circuit. The left femoral artery was identified, encircled with suture and cannulated with a 22G catheter which served as the arterial outflow for the CPB circuit. In the sham operation group the multi-orificed *iv* catheter was advanced only half its length in order to avoid effects on the heart.

Experimental protocol

Figure 1 shows the time course for the experimental protocol. Before initiating CPB, 10 mL of blood was withdrawn from animals in the Hct 10% group, and replaced with 5 mL Ringer's lactate and 5 mL

FIGURE 1 Schematic representation of the experimental protocol. CPB = cardiopulmonary bypass.

6% HAES-sterile. Thereafter, all animals in the three target Hct groups were cooled to a rectal temperature of 16°C to 18°C over 20 min, at which point CPB and ventilation were discontinued. Temperature was maintained by surface cooling for 90 min. Cardiopulmonary bypass was resumed after 90 min of arrest, and animals were actively warmed over 30 min via the heat exchanger to achieve a body temperature of 37°C. During the re-warming period the rat lungs were ventilated with 100% O₂ and dopamine 1 mg·kg⁻¹ was administered. After animals were then weaned from CPB, the left femoral arterial cannulae were removed and 60 min later, the rats were sacrificed. Brain tissue was dissected and prepared for further processing.

Histopathologic and immunohistochemistry analysis

After HCA and CPB (or after the same time lapse in the sham operation group), six rats of each group were perfused transcardially with 4% paraformaldehyde and phosphate-buffered saline (PBS) solution (stored at 4°C) for four to five minutes after a brief saline wash. Animals were then decapitated and the brains were dissected and cut into two hemispheres along the sagittal midline. The left hemisphere was immersed into a 2.5% glutaral and PBS solution (stored at 4°C) for ≥ 24 hr and then prepared for electron microscopy. The right hemisphere was immersed in a 4% formalin and PBS solution (stored at 4°C) for ≥ 24 hr until further processing. Later, coronal brain blocks were prepared for paraffin embedding. Coronal sections of 5 µm thickness were cut and stained with hematoxylin and eosin for histopathological analysis.

TABLE I Primers utilized for reverse transcriptase polymerase chain reaction

Primer	Primer sequence (5'-3')	Predicted size (base pair)	Cycle no.	Genbank accession
C-Fos				
Forward	5'-CCT CGA GGG GTT CCC GTA GA -3'	495	27	XM_234422
reverse	5'-ACA GTA CGT GGA TAT AGC GA-3'			
Bcl-2				
Forward	5'-CTGGTGGACAACATCGCTCTG-3'	228	29	NM_016993
reverse	5'-GGTCTGCTGACCTCACTTG TG-3'			
Bax				
Forward	5'-TCCAGGATCGAGCAGA-3'	256	29	NM_017059
reverse	5'-AAGTAGAAGAGGGCAACC-3'			
β -actin1				
Forward	5'-CCTCTATGCCAACACAGTGC-3'	211	27	NM_031144
reverse	5'-GTACTCCTGCTTGCTGATCC-3'			
β -actin2				
Forward	5'-ATTGTGACCAACTGGGACG-3'	533	29	NM_031144
reverse	5'-TTGCCGATAGTGATGACCT-3'			

As the predicted size of the β -actin1 was similar to that of Bcl-2 and Bax, β -actin2 was used to detect these two genes.

Coronal sections of six rats were prepared for immunohistochemistry analysis, performed using an avidin-biotin peroxidase method as described previously.¹⁵ Briefly, sections were washed in 0.1 mol·L⁻¹ PBS, incubated in 0.1 mol·L⁻¹ PBS containing 1% bovine albumin and 0.2% Triton-X-100 for 30 min, and subsequently incubated overnight with specific first antibody (C-Fos, Sigma, St. Louis, USA; 1:10000 dilution; Bcl-2, Sigma, 1:1000 dilution; Bax, Sigma, 1:5000 dilution). The next day, the brain sections were incubated with appropriate secondary IgG (1:200 dilution) for one hour, and with avidin-biotin peroxidase for one hour in a humidified chamber. PBS (0.1 mol·L⁻¹, pH 7.4) containing 0.5% bovine albumin was used to wash sections on slides between all steps. The antigen-antibody complexes were visualized by incubation for five minutes in 0.05% 3,3'-diaminobenzidine and 0.003% H₂O₂.

With the investigator blinded to group assignment, the sections were examined by standard light microscopy. Images were digitized with a video camera, and the number of injured neurons (stained with hematoxylin and eosin, the normal structures of the nucleus disappeared and were displaced with a blue mass without any nuclear structure), or the number of C-Fos, Bcl-2 and Bax immunoreactive cells in hippocampus CA1, parietal cortex and thalamus within the fixed 109634 pixels were measured using image-analysis software (DMBL QWin, Leica, Switzerland).

Electron microscopy

Ultra-thin sections of hippocampus CA1 were prepared from the left cerebral hemispheres immersed in the glutaral. Under transmission electron microscopy (HITACHI H-500, Tokyo, Japan), the investigator blinded to group assignment classified the mitochondrial injury into five scores (0–4) as follows: 0) normal mitochondria, full of granules; 1) normal structure but reduced granules; 2) swelling and matrix transparency; 3) cristae broken and matrix compact or transparent; 4) cristae broken and the inner membrane or outer membrane broken too. Five pictures of visual fields were randomly chosen under electron microscopy. Ten mitochondria in each picture were scored and the sum of scores was designated as the score of the picture. The mean score of five pictures was designated as the score of a specimen.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Rat brains were dissected and the hippocampus was separated and stored in liquid nitrogen for further processing. Total tissue RNA was extracted with the Trizol solution (Invitrogen Co., Tokyo, Japan) and subjected to the subsequent semi quantitative RT-PCR. The reverse transcription was performed in a total volume of 20 μ L comprising total RNA 1 μ g, 5 $\times$ Buffer 4 μ L, 10 μ M deoxy-ribonucleoside triphosphate 2 μ L, 50 μ M Oligo dT Primer 1 μ L, 40 U· μ L⁻¹

RNAse inhibitor (Promega Co., Madison, WI, USA) 0.5 μL , 200 U· μL^{-1} M-MLV RT enzyme (Promega Co., USA) and RNA-free H_2O 1 μL . Taking 10 μL of the reverse transcription product as a template, the PCR amplification was performed under conditions of 94°C for 180 sec, 60°C for 30 sec, 72°C for 300 sec, 27 cycles for C-Fos, and 29 cycles each for Bcl-2 and Bax. The quantities of template and cycle were examined and found to be within a line figure limit. The PCR products were separated on agarose gels and visualized by ethidium bromide staining and ultraviolet transillumination. Using two kinds of β -actin as an internal control, final numeric values were expressed as a ratio of the gene of interest to β -actin. The primer pairs for the target genes are listed in Table I.

Statistics analysis

Physiologic variables were assessed using a two-way analysis of variance to test for group-time interaction, followed by the LSD test for post hoc analysis when a significant interaction was identified. The histological and immunohistochemistry data and the scores of the mitochondrial injury were compared by analysis of one-way variance followed by the LSD test for post hoc analysis. Statistical analysis was performed with SPSS version 10.0, (SPSS Inc., Chicago, IL, USA) and all data are presented as the means $\pm$ SD. Statistical significance was assumed when $P < 0.05$.

Results

Blood gas analyses

Mean Hct values were similar amongst groups prior to initiation of CPB. While on CPB, Hct values decreased to the desired levels according to protocol in the target Hct groups. The re-warming period was associated with significant reductions of blood pH values in all groups, while the mean pH in the Hct 30% group was significantly greater than that observed in the Hct 20% group ($P < 0.05$) and the Hct 10% group ($P < 0.05$). At the start of re-warming, blood lactate concentrations were highest in the Hct 10% group and lowest in the Hct 30% group ($P < 0.05$). At other time points during CPB, the values of blood pH and blood lactic acid were not different amongst the three target Hct groups. The PaO_2 and SjvO_2 values were similar in all groups throughout the study (Table II).

Histopathology and immunohistochemistry analysis

Microscopy with hematoxylin and eosin staining demonstrated many injured neurons in the hippocampus CA1, parietal cortex and thalamus in the three target Hct groups, but not in the sham operation group. The number of injured neurons in the hippocampus

CA1 and parietal cortex was highest in the Hct 10% group and lowest in the Hct 30% group ($P < 0.05$). However, there was no difference in the number of the injured neurons in the thalami amongst the three experiment groups. Similarly, the numbers of C-Fos and Bax immunoreactive cells in the hippocampus CA1 and parietal cortex were greatest in the Hct 10% group and the lowest in Hct 30% group ($P < 0.05$). The number of Bcl-2 immunoreactive cells was the highest in the Hct 30% group and lowest in the Hct 10% group ($P < 0.05$), while no differences were observed in the thalami (Table III, Figure 2). No immunoreactive neurons were found in the brain sections of the sham operation group.

Ultramicroscopy analysis

The ultramicroscopic changes could be found very clearly under transmission electron microscopy. In the sham operation group neurons were rich in cytoplasm, polyribosomes and rough endoplasmic reticulum. The nuclei were nearly round; nuclear membranes and chromatospherites were clearly discernible, heterochromatin had the characters of neurons; a few neurofilaments could be found within the cytoplasm, and the mitochondria were normal.

In the Hct 10% group, neuronal heterochromatin in the hippocampus CA1 was condensed and the mitochondria were grossly swollen. Mitochondrial outer membranes were disrupted, and the mitochondrial injury scores ranged between 3 and 4.

In the Hct 20% group, the nuclear membranes of neurons in the hippocampus CA1 were shrunken. Mitochondria were moderately swollen, cristae were disrupted, but granules still could be observed on the intact mitochondrial outer membrane. Mitochondrial injury scores ranged between 2 and 3. In the Hct 30% group, much cytopharyngeal apparatus could be found intact within the cytoplasm. Mitochondria were slightly swollen; parts of cristae were disrupted and the number of granules was decreased. Mitochondrial injury scores in this group ranged between 1 and 2.

The results of the image analysis indicated that the mean mitochondrial injury scores were highest in the Hct 10% group and lowest in the Hct 30% group ($P < 0.05$), Figure 3, Table IV).

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Hypothermic circulatory arrest (HCA) and CPB induced the transcription of Bax, Bcl-2 and C-Fos mRNA in the hippocampus of the hemodiluted rats (Figure 4). Semi-quantitative measurement (ratio of mRNA transcription of genes of interest to that

TABLE II Physiologic variables (mean $\pm$ SD)

	<i>Hct 10% group (I)</i>	<i>Hct 20% group (II)</i>	<i>Hct 30% group (III)</i>	<i>I vs II</i>	<i>P value I vs III</i>	<i>II vs III</i>
Hct (%)						
Preop	42.2 $\pm$ 2.3	41.3 $\pm$ 2.2	42.0 $\pm$ 2.6	NS	NS	NS
0 min	9.5 $\pm$ 1.1	19.7 $\pm$ 1.8	30.3 $\pm$ 1.6	< 0.05	< 0.05	< 0.05
110 min	11.2 $\pm$ 2.1	20.5 $\pm$ 1.9	30.5 $\pm$ 1.9	< 0.05	< 0.05	< 0.05
pH						
0 min	7.355 $\pm$ 0.058	7.400 $\pm$ 0.051	7.396 $\pm$ 0.050	NS	NS	NS
20 min	7.373 $\pm$ 0.015	7.385 $\pm$ 0.036	7.416 $\pm$ 0.059	NS	NS	NS
110 min	6.693 $\pm$ 0.182	7.030 $\pm$ 0.169	7.250 $\pm$ 0.108	< 0.05	< 0.05	< 0.05
140 min	7.375 $\pm$ 0.038	7.370 $\pm$ 0.042	7.380 $\pm$ 0.040	NS	NS	NS
PO ₂ (kPa)						
0 min	292 $\pm$ 12	289 $\pm$ 19	275 $\pm$ 4	NS	NS	NS
20 min	284 $\pm$ 7	285 $\pm$ 12	284 $\pm$ 11	NS	NS	NS
110 min	286 $\pm$ 3	289 $\pm$ 6	285 $\pm$ 9	NS	NS	NS
140 min	287 $\pm$ 7	278 $\pm$ 2	282 $\pm$ 10	NS	NS	NS
SO ₂ (%)						
0 min	98.9 $\pm$ 0.7	99.1 $\pm$ 0.8	99.2 $\pm$ 0.5	NS	NS	NS
20 min	99.2 $\pm$ 0.5	99.1 $\pm$ 0.5	99.0 $\pm$ 0.8	NS	NS	NS
110 min	99.1 $\pm$ 0.8	99.1 $\pm$ 0.9	99.2 $\pm$ 0.5	NS	NS	NS
140 min	99.0 $\pm$ 0.9	99.3 $\pm$ 0.5	99.0 $\pm$ 0.8	NS	NS	NS
SjvO ₂ (%)						
0 min	76.8 $\pm$ 0.9	77.2 $\pm$ 0.8	76.8 $\pm$ 1.1	NS	NS	NS
20 min	97.1 $\pm$ 1.0	97.2 $\pm$ 0.5	97.0 $\pm$ 0.6	NS	NS	NS
110 min	47.6 $\pm$ 2.0	48.1 $\pm$ 1.5	47.1 $\pm$ 1.9	NS	NS	NS
140 min	77.1 $\pm$ 0.6	77.1 $\pm$ 0.8	76.9 $\pm$ 1.1	NS	NS	NS
Lac (mmol·L ⁻¹)						
0 min	4.53 $\pm$ 0.34	4.67 $\pm$ 0.31	4.72 $\pm$ 0.44	NS	NS	NS
20 min	4.98 $\pm$ 0.26	4.85 $\pm$ 0.19	4.83 $\pm$ 0.34	NS	NS	NS
110 min	22.70 $\pm$ 1.36	12.93 $\pm$ 1.86	8.47 $\pm$ 1.30	< 0.05	< 0.05	< 0.05
140 min	4.92 $\pm$ 0.52	4.85 $\pm$ 0.24	4.72 $\pm$ 0.31	NS	NS	NS

Hct = hematocrit; PO₂ = arterial oxygen pressure; SO₂ = arterial oxygen saturation; SjvO₂ = oxygen saturation of jugular venous blood; Lac = blood lactic acid; Preop = preoperative; NS = not significant.

TABLE III The numbers of injured neurons and C-Fos, Bcl-2 and Bax immunoreactive cells in the hippocampus CA1, parietal cortex and thalamus (mean $\pm$ SD; cells/109634 pixels)

	<i>Hippocampus CA1</i>	<i>Parietal cortex</i>	<i>Thalamus</i>
<i>Histopathology</i>			
Hct 10% group	11.44 $\pm$ 2.52*	13.65 $\pm$ 2.31*	7.05 $\pm$ 3.01
Hct 20% group	8.29 $\pm$ 1.31*	10.68 $\pm$ 1.24*	6.26 $\pm$ 2.41
Hct 30% group	4.40 $\pm$ 1.98*	7.38 $\pm$ 2.11*	4.42 $\pm$ 1.94
<i>Bax</i>			
Hct 10% group	22.50 $\pm$ 1.87*	24.33 $\pm$ 3.88*	10.50 $\pm$ 1.87
Hct 20% group	14.67 $\pm$ 2.94*	14.00 $\pm$ 2.83*	10.00 $\pm$ 2.61
Hct 30% group	10.83 $\pm$ 1.47*	9.17 $\pm$ 1.72*	9.17 $\pm$ 1.72
<i>Bcl-2</i>			
Hct 10% group	9.33 $\pm$ 1.21	9.67 $\pm$ 1.03*	10.83 $\pm$ 1.47
Hct 20% group	11.00 $\pm$ 1.41	11.83 $\pm$ 2.04*	9.50 $\pm$ 1.87
Hct 30% group	13.33 $\pm$ 2.16*	15.50 $\pm$ 1.87*	10.50 $\pm$ 1.87
<i>C-Fos</i>			
Hct 10% group	14.00 $\pm$ 2.45*	18.67 $\pm$ 2.16*	9.33 $\pm$ 2.16
Hct 20% group	10.83 $\pm$ 1.94*	12.50 $\pm$ 1.87*	10.33 $\pm$ 2.07
Hct 30% group	5.83 $\pm$ 0.75*	7.50 $\pm$ 1.05*	8.00 $\pm$ 0.89

Hct = hematocrit. Histopathology: stained with hematoxylin and eosin and the injured neurons were counted with an image-analysis system. * P < 0.05, different from the other two groups by ANOVA and the LSD post-hoc test.

of an internal gene, β -actin) is shown in Table V. Hippocampal transcription of Bax, Bcl-2 and C-Fos increased after HCA in the hemodiluted rats compared with the sham operation group (P < 0.05). Transcription of the C-Fos and Bax mRNA was highest in Hct 10% group and lowest in the Hct 30% group (P < 0.05). However, transcription of the Bcl-2 mRNA was highest in the Hct 30% group (P < 0.05).

Discussion

Hypothermic circulatory arrest is a widely adopted technique in congenital heart operations and aortic arch or thoracic aortic procedures.^{16–20} Although many improvements have been introduced since the technique was first established, there remain recognized complications secondary to unique physiologic trespasses associated with HCA and CPB.^{19–22} Further refinements of the technique require an appropriate experimental model. In the present study, we developed a HCA model using rats, which was designed to mirror clinical circulatory arrest to the greatest extent

FIGURE 2 Bax, Bcl-2 and C-Fos immunoreactive neurons in the parietal cortex of the three target hematocrit (Hct) groups. Panels A, B, C = Bax immunoreactive neurons in the Hct 10%, Hct 20% and Hct 30% groups respectively. Panels D, E, F = Bcl-2 immunoreactive neurons in the Hct 10%, Hct 20% and Hct 30% groups respectively. Panels G, H, I = C-Fos immunoreactive neurons in the Hct 10%, Hct 20% and Hct 30% groups respectively. The numbers of C-Fos and Bax immunoreactive neurons were greatest in the Hct 10% group and the lowest in the Hct 30% group ($P < 0.05$). The number of Bcl-2 immunoreactive neurons was the highest in the HCT 30% group and lowest in the HCT 10% group ($P < 0.05$). The number of C-Fos, Bax and Bcl-2 immunoreactive neurons were similar in the thalami in all three experiment groups.

possible.¹¹ As with other related rodent models,^{23,24} we used chloral hydrate as the primary anesthetic. Our findings provide convincing evidence that low Hct worsens cerebral injury after prolonged HCA.

Our results are generally consistent with other reports. Hare *et al.*²⁵ demonstrated that anesthetized rats hemodiluted to a hemoglobin concentration of $5.1 \text{ g}\cdot\text{dL}^{-1}$ sustained a significant decrease in tissue oxygen tension. Using a piglet model, Sakamoto *et al.*²⁶ showed that higher Hct values (30%) could minimize brain damage after HCA. In a rabbit model Reasoner *et al.*²⁷ found that marked hemodilution exacerbates neurological injury resulting from permanent focal cerebral ischemia.

Habib *et al.*¹⁰ have demonstrated in humans that the incidence of stroke, myocardial infarction, low cardiac output syndrome, cardiac arrest, renal failure, pulmonary edema, re-operation due to bleeding, sepsis, and multi-organ failure all significantly increase as Hct values decrease below 22%. Consequently, intensive care requirements, hospital length of stay, operative costs, and perioperative mortality were also significantly greater at progressive levels of hemodilution. Longer-term survival has been shown to be greater in association with increasing Hct during coronary artery bypass grafting.²⁸ This suggests that long-term changes in brain tissue following HCA and CPB may be initiated as a result of hemodilution. The nature of these changes at the cellular and molecular levels have yet to be elucidated. The initiation of an apoptotic process that results in delayed loss of neurons may be one possible explanation to account for the occurrence of these long-term neuronal injuries.

Apoptosis is a well documented series of events that results in the programmed self-destruction of cells,²⁹ which is an important mechanism of neuronal death.

FIGURE 3 Ultramicroscopic changes of the hippocampus CA1 after hypothermic circulatory arrest ($\times 20000$). Panel A = hematocrit (Hct) 10% group, mitochondria swollen with the outer membrane disrupted; Panel B = Hct 20% group, mitochondria moderately swollen, cristae disrupted or absent but an intact mitochondrial outer membrane is observed; Panel C = Hct 30% group, mitochondria slightly swollen; cristae and granules still can be observed on the intact mitochondrial outer membrane.

TABLE IV Scores of the mitochondrial injury (mean $\pm$ SD)

	Control (n = 4)	Hct 10% (n = 4)	Hct 20% (n = 4)	Hct 30% (n = 4)
Mitochondrial injury score	0	34.05 $\pm$ 1.56*	29.30 $\pm$ 0.68*	18.65 $\pm$ 0.53*

Hct = hematocrit. * $P < 0.05$, different from the other two groups by ANOVA and the LSD post-hoc test.

TABLE V Changes of the transcription of Bax, Bcl-2 and C-Fos mRNA in the hippocampus CA1 after hypothermic circulatory arrest (mean $\pm$ SD)

	Bax	Bcl-2	C-Fos
Sham operation group	0.74 $\pm$ 0.10*	0.53 $\pm$ 0.10*	0.97 $\pm$ 0.19*
Hct 10% group	1.41 $\pm$ 0.12*	0.75 $\pm$ 0.12	1.94 $\pm$ 0.19*
Hct 20% group	1.22 $\pm$ 0.14*	0.76 $\pm$ 0.10	1.66 $\pm$ 0.13*
Hct 30% group	0.99 $\pm$ 0.14*	0.97 $\pm$ 0.04*	1.33 $\pm$ 0.16*

Hct = hematocrit. * $P < 0.05$, different from the other two groups by ANOVA and the LSD post-hoc test.

Balanced expressions between the pro-apoptotic (e.g., Bax, Bcl-x and Bad) and anti-apoptotic (e.g., bcl-2, ced-9) genes ultimately results in either cell survival or apoptosis.³⁰ The initiation of apoptotic processes may explain the differential neurological responses to different levels of hemodilution after HCA and CPB.^{31,32}

Krajewska *et al.*³³ utilized a canine cardiac arrest model of global cerebral ischemia to demonstrate the spatial and temporal patterns of expression and proteolytic processing of Bcl-2-family proteins. Sato *et al.*³⁴ found in the rat that after 90 min of CPB, Bax, and Bcl-2 were significantly increased. Kin *et al.*³² observed from a piglet CPB model that neuronal cell death observed in CA1 pyramidal cells 72 hr after 90 min of deep hypothermic circulatory arrest at 15°C results in apoptosis. It has been shown in New Zealand white rabbits that apoptosis occurred specifically in hippocampal CA1 neurons after a two-hour hypothermic circulatory arrest followed by four hours of reperfusion.³⁵

We recently reported our experience with a rat model of CPB that allows for the investigation of molecular biological changes in the brain.¹¹ In this study we observed that the transcription and the translation of the Bcl-2 and Bax mRNA increased after HCA. However, the transcription and the translation of the anti-apoptotic Bcl-2 mRNA in the Hct 30% group was significantly greater than that observed in the Hct 10% and Hct 20% groups, while the transcription and translation of the pro-apoptotic Bax mRNAs was lowest in the Hct 30% group and highest in the Hct 10% group. These findings indicate that regulat-

FIGURE 4 Changes of Bax, Bcl-2 and C-Fos mRNA in the hippocampus after hypothermic circulatory arrest (HCA). Lane M = marker; lane 1 = sham operation group; lane 2 = hematocrit (Hct) 10% group; lane 3 = Hct 20% group. Lane 4 = Hct 30% group. Hippocampal expression of Bax, Bcl-2 and C-Fos mRNAs increased after HCA in the hemodiluted rats as compared with the sham operation group ($P < 0.05$). Expression of the pro-apoptotic C-Fos and Bax mRNA was highest in the Hct 10% group and lowest in the Hct 30% group ($P < 0.05$). In contrast, expression of the anti-apoptotic Bcl-2 mRNA was highest in the Hct 30% group ($P < 0.05$). (Because of the similar molecular weights of C-Fos and β -actin1, in the C-Fos experiment β -actin2 was substituted for C-Fos).

ing the balance of expression of pro-apoptosis and anti-apoptosis genes is important in explaining the differential effects of hemodilution on the brain after HCA and CPB.

The molecular events initiated as a result of CPB are only beginning to be understood. Bokesch *et al.*³⁶ first described the transcription of C-Fos genes resulting from a combination of CPB and global cerebral ischemia induced during hypothermic circulatory arrest in lambs. In their study, they described up-regulation of C-Fos mRNA, whose protein products function as transcription regulators of genes affecting cell survival

and death. The magnitude of C-Fos gene expression increased linearly with the duration of HCA, with maximum expression after 120 min of HCA. Intense Fos-like immunoreactivity was detected in neurons of the hippocampal formation in animals subjected to 90 min of HCA.

Just as Bokesch³⁶ reported in his study, we also found that after 90 min HCA and CPB, the transcription and translation of the C-Fos mRNA in three target Hct groups increased significantly compared with the control group. However, the magnitude of C-Fos gene expression was highest in the Hct 10% and lowest in the Hct 30% group. In rodents, C-Fos expression by CA1 neurons of the hippocampus after global ischemia is associated with the subsequent degeneration of these neurons and the C-Fos mRNA and protein were markers of brain injury,^{32,34–36} therefore regulating the expression of C-Fos may be another molecular explanation that low Hct worsens cerebral injury after HCA and CPB.

This study has several limitations. First, the transcription of apoptotic genes into their respective mRNAs and subsequent translation into proteins is only a preliminary step in the complete process of apoptosis.^{37,38} Although we demonstrated that mRNAs encode for both pro-apoptotic and anti-apoptotic pathways expressed after 90 min of HCA and CPB, the more important step along the apoptotic pathway has not been elucidated, and further studies are required. Secondly, in this study, there was no control group of animals who were subjects to CPB alone; accordingly, the observed cellular injury occurred at low Hct secondary to both HCA and CPB. Finally, it has been shown that brain histopathologic changes are not observed routinely after 80 min of HCA.³⁹ Accordingly, the period of circulatory arrest was set at 90 min to ensure that the intervention would achieve a measurable response. We acknowledge that this duration is longer than the usual upper limit of circulatory arrest permitted in humans (40 min)⁴⁰ and warrants caution in extrapolating these observations to the clinical setting.

In conclusion, we have demonstrated in a rat model of HCA and CPB that low Hcts result in brain damage which might be explained, in part, by variable expression of the C-Fos, Bcl-2 and Bax genes. The effects of cerebral edema, low perfusion pressure, and low colloid oncotic pressure related to hemodilution may be additional contributing factors. Further studies are warranted to determine the role of these variables on neuronal injury. This knowledge may eventually lead to the development of targeted strategies to improve postoperative neurological outcome associated with HCA and CPB.

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