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Upregulation of hypoxia inducible factor is associated with attenuation of neuronal injury in neonatal piglets undergoing deep hypothermic circulatory arrest

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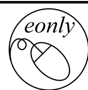

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Background: Prolonged deep hypothermic circulatory arrest is known to cause neurological injury. Hypoxia inducible factor, a transcription factor that mediates adaptive changes during hypoxia, is neuroprotective in models of ischemic brain injury, in part by upregulating erythropoietin. This study tested the hypothesis that upregulation of hypoxia inducible factor and erythropoietin by preconditioning with hypoxia or the hypoxia-mimetic agents deferoxamine and cobalt chloride would be neuroprotective in a piglet model of deep hypothermic circulatory arrest.

Methods: Anesthetized neonatal piglets were randomized to 4 preconditioning groups (15 per group): hypoxia, deferoxamine, cobalt chloride, or control (NaCl vehicle). Brain hypoxia inducible factor and erythropoietin contents were assessed by means of Western blotting at 3, 8, and 24 hours after treatment ($n = 3$ per time point). Twenty-four hours after treatment, 6 to 7 animals per group underwent cardiopulmonary bypass and 110 minutes of deep hypothermic circulatory arrest. After recovery, serial neurobehavioral examinations were conducted for 6 days, after which histopathologic brain injury and neuronal apoptosis (cleaved caspase 3) were assessed.

Results: Erythropoietin expression was not significantly increased by any of the pretreatment strategies. In contrast, there was a significant upregulation of hypoxia inducible factor by pretreatment with deferoxamine and cobalt chloride ($P = .002$). Neurobehavioral measures revealed no significant differences in time to recovery or extent of injury. Examination of histopathologic brain injury in the hippocampus revealed that pretreatment with deferoxamine (0.4 ± 0.3) and cobalt chloride (0.5 ± 0.3) were associated with significantly less neuronal loss than pretreatment with hypoxia or control (2.8 ± 0.5, $P = .004$). Finally, cleaved caspase 3 (a marker of apoptotic cell death) was also shown to be diminished in the cobalt and deferoxamine groups, but the difference was not significantly different from the value in the control group.

Conclusions: In contrast to hypoxia, deferoxamine and cobalt chloride preconditioning upregulated hypoxia inducible factor and were associated with histopathologic neuroprotection after exposure to cardiopulmonary bypass and prolonged deep hypothermic circulatory arrest.

CHD

Deep hypothermic circulatory arrest (DHCA) is often used during repairs of complex congenital cardiac defects. However, many patients undergoing DHCA have neurodevelopmental abnormalities that are manifested days or even years after recovery from the surgical procedure. These abnormalities range from subtle subclinical radiographic findings to overt cognitive and functional impairment, including seizures, choreoathetosis, and impaired scoring on psychomotor developmental indices.[1,2]

Hypoxia inducible factor (HIF) is a transcription factor that mediates adaptive mechanisms during periods of hypoxic stress. It is a heterodimeric protein consisting of 2 subunits, HIF-1α and HIF-1β. HIF-1β is constitutively expressed in the nucleus and maintains a relatively constant level. In contrast, HIF-1α is rapidly ubiquinated in the cytoplasm and removed by means of proteasomal degradation during periods of normal oxygen tension. However, during hypoxic conditions, the degradation of HIF-1α is blocked, allowing it to accumulate within the cell. The increased intracellular concentration of HIF-1α then allows it to translocate to the nucleus, where it forms a heterodimer with HIF-1β and becomes an active transcription factor for several key end products that are important for anaerobic metabolism and cell survival, including erythropoietin (EPO), vascular endothelial growth factor, glucose transporter enzymes, and glycolytic enzymes.[3]

Similar in concept to ischemic preconditioning, which has been widely studied as an approach to alleviate ischemia-reperfusion injury in the heart, hypoxic preconditioning has been investigated as a protective strategy preceding permanent cerebral ischemia in rodents.[4-7] In these rodent models of brain infarction, animals exposed to antecedent hypoxia or hypoxia-mimetic agents (deferoxamine or cobalt chloride [$CoCl_2$]) were more tolerant of subsequent cerebral ischemia, a benefit attributed to the upregulation of HIF-1α[4-6] and its target gene products. Therefore on the basis of these previous studies, we hypothesized that upregulation of HIF-1α and EPO before a period of DHCA in neonatal piglets would have similar neuroprotective benefits by alleviating the injury resulting from the inherent global ischemia encountered during circulatory arrest.

Methods

Surgical Preparation

Animal care was conducted under the approval of the Institutional Animal Care and Use Committee of Emory University and in compliance with the "Guiding Principles in the Use and Care of Animals," as published by the National Institutes of Health in 1996. All surgical procedures were carried out under sterile conditions.

This study was completed in 2 phases: the goal of phase I was to determine whether HIF-1α and EPO could be upregulated in piglets by stimuli similar to those that had been previously used in rodent models. In this part of the study, 36 2- to 3-week-old Yorkshire cross piglets were preanesthetized with an intramuscular cocktail of ketamine (22 mg/kg), acepromazine (1.1 mg/kg) and atropine (0.05 mg/kg) and then orotracheally intubated and ventilated with a pediatric veterinary ventilator (Model 2000; Hallowell, Inc, Pittsfield, Mass) to maintain pH at a goal of 7.35 to 7.45, Pco_2 at 35 to 45 mm Hg, and Po_2 at 100 to 110 mm Hg. Deep anesthesia was maintained with 1% to 1.5% inhaled isoflurane. A marginal ear vein was cannulated for administration of drugs and intravenous fluids, and the superficial femoral artery was cannulated with a 22-gauge fluid-filled catheter for monitoring of blood pressure, heart rate, and arterial blood gases. The animals were then randomly assigned to one of 4 groups: (1) control (ventilated with supplemental oxygen to maintain an arterial Po_2 of 95-125 mm Hg for 3 hours and received an intravenous and intraperitoneal injection of 0.225% NaCl as a vehicle); (2) hypoxia (ventilated with a hypoxic gas mixture to maintain an arterial Po_2 of 30-40 mm Hg for a period of 3 hours and received an intravenous and intraperitoneal injection of 0.225% NaCl); (3) deferoxamine (maintained arterial Po_2 of 95-125 mmHg and received an intravenous [100 mg/kg] and intraperitoneal [100 mg/kg] injection of deferoxamine, a hypoxia-mimetic agent); and (4) $CoCl_2$ (maintained arterial Po_2 of 95-125 mm Hg and received an intravenous injection of 0.225% NaCl and intraperitoneal [20 mg/kg] injection of $CoCl_2$, a hypoxia-mimetic agent). The doses of $CoCl_2$ and deferoxamine were chosen on the basis of previous doses used in similar rodent studies. (Cobalt has been safely administered in rodents up to 60 mg/kg; however, in the current study such high doses caused hemodynamic collapse and death. Therefore a smaller dose was used that did not seem to have significant adverse effects. We did not, however, perform a formal dose-response study to determine the extent of HIF-1α upregulation by greater doses of deferoxamine and $CoCl_2$.) The animals were then euthanized at 3, 8, and 24 hours ($n = 3$ in each group) after the start of the experiment with an overdose of pentobarbital (100 mg/kg administered intravenously). The brains of these animals were then removed en bloc, snap-frozen in liquid nitrogen, and stored in a freezer at $-80°C$ until ready for Western blot analysis of HIF-1α and EPO.

The goal of phase II was to determine whether HIF-1α and EPO would be neuroprotective in piglets subjected to DHCA. In this phase 25 piglets ($n = 6$-7 in each group) underwent the same preconditioning treatments as described above (ie, 3 hours of anesthesia, either maintaining hypoxia or normoxia with injection of deferoxamine, $CoCl_2$, or vehicle). However, these animals were allowed to recover for 24 hours, at which time they were reanesthetized in a manner similar to that described above. Cefazolin (25 mg/kg administered intravenously) was administered before the procedure on each day and every 8 hours thereafter for a period of 24 hours. Deep anesthesia was maintained with 1% to 1.5% inhaled isoflurane, except during the period of DHCA. Once again, the superficial femoral artery was cannulated for monitoring purposes. After systemic heparinization (300 U/kg), the contralateral common femoral artery was cannulated with an 8F arterial cannula (Bio-Medicus, Minneapolis, Minn), and the right atrial appendage was cannulated with an 18F venous cannula (Baxter RMI, Deerfield, Ill) through a right anterolateral thoracotomy.

The cardiopulmonary bypass (CPB) circuit consisted of a nonpulsatile roller pump (Cobe Cardiovascular, Inc, Arvada, Colo), sterile tubing, a pediatric membrane oxygenator (Lilliput 2; Cobe Cardiovascular, Inc, Arvada, Colo), a venous reservoir (Lilliput 1 twin reservoir, Cobe Cardiovascular, Inc), and a 40-μm arterial filter (Cobe Cardiovascular, Inc). The circuit was primed with 500 mL of whole porcine blood (Lampire Biological Labs, Pipersville, Pa), 1000 U of heparin, 30 mg/kg calcium chloride, 15 mEq sodium bicarbonate, 1 g/kg mannitol, 5 mg/kg dexamethasone, and 0.1 mg/kg pancuronium bromide.

The animals were started on CPB and perfusion cooled to a nasopharyngeal temperature of 18°C over a period of 20 minutes,

followed by a 110-minute period of circulatory arrest. Perfusion was initiated at a flow rate of 150 mL/kg and adjusted to maintain a perfusion pressure of greater that 30 mm Hg. During the cooling and rewarming periods, hematocrit was maintained at or near 30%, and arterial blood gases were managed according to the alpha-stat strategy, maintaining a PCO_2 of 35 to 45 mm Hg uncorrected for temperature. The head was packed in ice during this time, and the heart was topically cooled and arrested without the use of cardioplegia. During the circulatory arrest period, the venous cannula was unclamped periodically to completely drain the venous system of any accumulated blood. After reinstitution of CPB, the animals were rewarmed and weaned from CPB once they reached a temperature of 34°C. The animals were then decannulated, a 6F red rubber thoracostomy tube was placed, and all incisions were closed in multiple layers with absorbable sutures.

Postoperative Care

While the animals recovered from anesthesia, oxygen saturation and heart rate were continuously monitored with a pulse oximeter (Novametrix Medical Systems, Inc, Wallington, Conn). When the animals were able to breathe spontaneously, mechanical ventilation was discontinued. The endotracheal tube was removed when it was no longer tolerated, and the animals were moved to a temperature-controlled recovery kennel. The thoracostomy tube was removed when there was minimal output. The ear vein cannula was maintained until the animals were able to eat-drink independently. Animals incapable of eating-drinking were supported with gastric feeds through a nasogastric tube. Buprenorphine (0.1 mg/kg administered intravenously) was used for pain management in the immediate postoperative period. The animals were allowed to survive for 6 days, during which they underwent serial neurobehavioral assessments by a blinded observer according to a standardized scoring system (Table E1).

Perfusion Fixation of Brain

At the end of the 6-day survival period, the animals were sedated with an intramuscular cocktail of ketamine (22 mg/kg) and acepromazine (1.1 mg/kg). A marginal ear vein was cannulated for administration of systemic heparin (300 U/kg) and euthanasia solution (sodium pentobarbital, 100 mg/kg). The right carotid artery was exposed and cannulated with a 14-gauge angiocatheter. The right chest was opened, and the superior vena cava was incised to allow exsanguination. Through the carotid artery, the brain was perfused in situ with 1 L of normal saline, followed by 1 L of 4% paraformaldehyde (dissolved daily in 0.1 mol/L sodium phosphate buffer) at a pressure of 100 mm Hg. After removal of the scalp and cranial vault, the brain was excised en bloc with the cerebellum and brain stem and placed in 10% buffered formalin for permanent fixation.

Preparation of Homogenates From Brain Tissue for Western Blot Analysis

The fresh brain was frozen immediately with liquid nitrogen and stored at −80°C until use to prepare total homogenates from porcine brain tissue. Small pieces of brain tissue were placed in homogenizing buffer (containing the following: Tris/HCl, 50 mmol/L [pH 7.5]; NaCl, 150 mmol/L; ethylenediamine tetraacetic acid, 1 mmol/L; dithiothreitol, 1 mmol/L; pepstatin A, 0.001 mmol/L; phenylmethylsulfonyl fluoride, 0.4 mmol/L; phenanthroline, 1 mmol/L; iodoacetamide, 1 mmol/L; aprotinin, 0.01 U/mL; and 0.1% Triton X-100) and homogenized with a Tekmar Tissumizer for 30 seconds and repeated several times until tissue granules could not be seen. Then the homogenate was stored at 4°C overnight for lysis and extraction of proteins from both cytoplasm and nuclei. The sample was then centrifuged at 3000*g* for 20 minutes, and the supernatant (total homogenate) was stored at −80°C in small aliquots. Protein was determined by using the Bradford dye method (BioRad, Hercules, Calif).

Western Blot Analysis for HIF-1α and EPO

HIF-1α and EPO levels were analyzed in total homogenates from whole brain tissue, as previously described,[8,9] by using a monoclonal antibody for HIF-1α (Calbiochem, San Diego, Calif) and a polyclonal antibody for EPO (Santa Cruz Biotechnology, Santa Cruz, Calif). For detection of bands, an enhanced chemiluminescence detection kit (ECL+plus, Amersham Corp, Piscataway, NJ) with lumigen PS-3 substrate was used. To compare relative amounts of specific proteins, we used a 2-dimensional gel imaging system and Labworks image acquisition and analysis software (UVP, Inc, Upland, Calif), with data presented as arbitrary optical density units.

Histopathologic Analysis and Immunostaining for Cleaved Caspase 3

After storage in 10% buffered formalin, brains from phase II animals were cut in the coronal plane, processed, and embedded in paraffin blocks. Six-micrometer sections were then mounted on slides for hematoxylin and eosin staining, as well as for immunostaining for caspase 3 and cleaved caspase 3 as a marker of apoptosis. Immunohistochemistry was performed on sections that were deparaffinized and subjected to heat-induced epitope retrieval by steaming for 15 minutes. Slides were then incubated at room temperature with antibodies directed toward caspase 3 (rabbit polyclonal, 1:25; Cell Signaling, Beverly, Mass) and cleaved caspase 3 (rabbit polyclonal, 1:100; Cell Signaling).

Immunostaining for caspase 3 and cleaved caspase 3 was performed in the hippocampus only because this region is known to be particularly susceptible to ischemic injury.[10] The number of total nuclei and apoptotic nuclei (as indicated by brown staining for cleaved caspase 3) were quantified at 20× magnification by using a computer-automated counting system and ImagePro 4.0 software (Media Cybernetics Inc, Silver Springs, Md). The percentage of apoptotic nuclei averaged from a minimum of 3 random fields is presented.

Hematoxylin and eosin–stained slides were examined by a neuropathologist (D.J.B.) blinded to the experimental groups. Seven regions of the brain were analyzed, including the frontal and parietal neocortex, hippocampus, striatum, cerebellum, thalamus, and pons. Each region was examined for 3 discrete changes: neuronal hypereosinophelia as a marker of acute hypoxic injury, reactive gliosis as an indicator of tissue response to injury, and frank tissue necrosis (infarction). Each region received a score ranging from 0 (no injury) to 4 (severe injury).

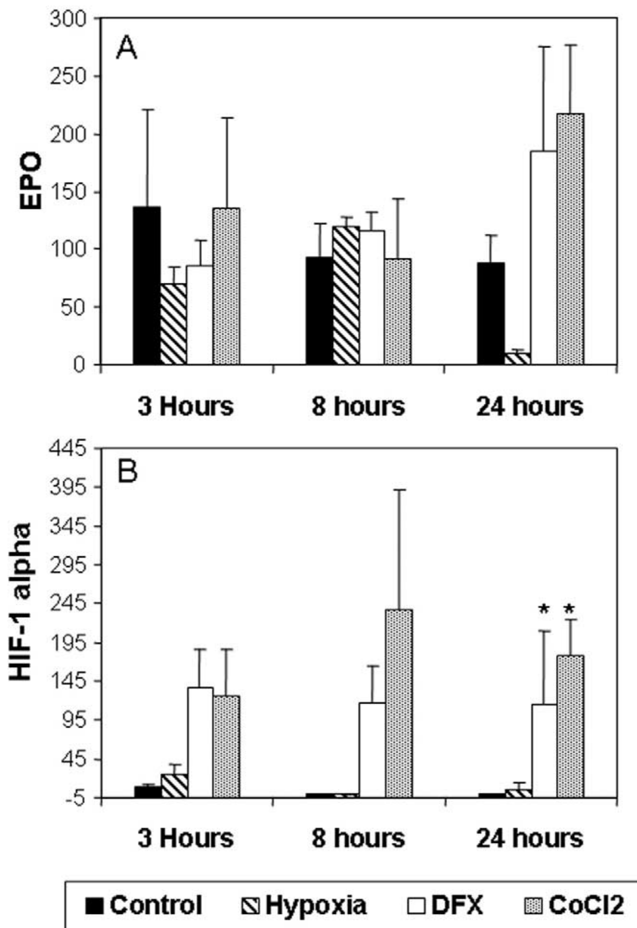

Figure 1. Brain content of erythropoietin (EPO; A) and hypoxia inducible factor (HIF-1α; B) at 3, 8, and 24 hours after preconditioning stimulus by means of Western blot assay. *$P < .05$. *DFX*, Deferoxamine; *$CoCl_2$*, cobalt chloride.

Statistical Analysis: Phase I

A nested general linear model was performed to test for overall group and time-within-group effects for both EPO and HIF-1α. For significant group effects, subsequent post-hoc Dunnett tests were conducted to compare treatment groups with the control group. For significant time-within-group effects, we performed appropriate tests of the estimable functions to determine the source of the overall effect. Because of the fact that many of the HIF-1α values were nondetectable (ie, 0), we also performed a nonparametric Kruskal-Wallis test of the group effect, as well as a test of the group effect on the proportion of detectable values.

Statistical Analysis: Phase II

Perioperative variables. We used 1-way analysis of variance to determine whether there were any significant group differences in biologic measures that would indicate procedural differences. This was done to determine any effects that might need to be controlled for in the testing of group effects on the main outcome variables.

Repeated neurobehavioral measurements. Because of the fact that many of the test animals eventually returned to baseline (score = 0), we compared the group, time, and group-by-time interaction on the proportion of impaired animals by using repeated-measures logistic regression (population-averaged generalized estimating equations [GEE] model).

Brain injury. We first performed tests of group effects in the data summed across brain region (7 types) and type of brain injury (neuronal loss, gliosis, and tissue necrosis) by using Kruskal-Wallis tests to determine initial group differences of interest. We then tested for group differences in the most significant regions and types by using ordinal logistic regression.

Apoptosis. On the basis of the results from the brain injury analysis, we looked at a marker of apoptotic cell death, caspase 3, within the brain regions that demonstrated significant injury. Because of the fact that the distribution of caspase 3 was somewhat skewed in most regions, we used nonparametric Kruskal-Wallis tests to test for significant group differences.

Results

Upregulation of HIF-1α and EPO by Hypoxic Preconditioning or Hypoxia-mimetic Agents

Western blot analyses of brains for content of HIF-1α and EPO were performed 3, 8, and 24 hours after the preconditioning stimulus. The mean values for EPO and HIF-1α are presented in Figure 1. Although there is a trend toward increased EPO expression in the deferoxamine and $CoCl_2$ groups at 24 hours, these results indicated no statistically significant upregulation of EPO by any of the pretreatment strategies (Figures 1, *A*, and E1,*A*).

In contrast, analysis of the HIF-1α data showed a significant increase, compared with that seen in the control group, in the deferoxamine (P = .026) and $CoCl_2$ (P = .002) groups but not in the hypoxia (P = .686) group. Although a test of means for these data is tenuous, the result was supported by the nonparametric test ($\chi^2 = 24.57$, $df = 3$, $P < .0005$), as well as the test of the proportion of nondetectables (control = 78%, hypoxia = 56%, deferoxamine = $CoCl_2$ = 0%, $\chi^2 = 19.00$, $df = 3$, $P < .0005$). Thus the data indicated a significant upregulation of HIF-1α by means of pretreatment with deferoxamine and $CoCl_2$ (Figures 1, *B*, and E1, *B*).

Phase II: Perioperative Variables

There were no significant group differences in perioperative variables (Table E2), and therefore no adjustments were made to the analyses of the primary outcomes. Mean arterial pressure and heart rate were similar in all groups before CPB and after recovery from DHCA, as were cooling and total CPB times. Arterial P_{CO_2} was maintained at 40 to 45 mm Hg before and after CPB, without significant differences between groups. Likewise, on-pump and post-CPB hematocrit values were similar in all groups and were maintained at a level of

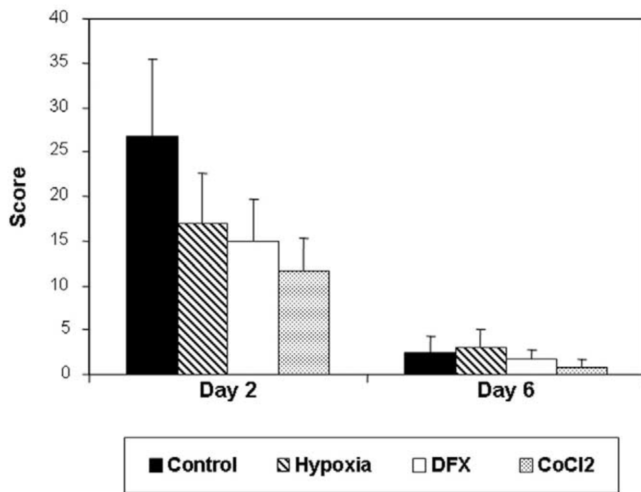

Figure 2. Neurobehavioral examination scores on postoperative days 2 and 6. Cobalt chloride–treated animals had diminished injury on postoperative day 2 compared with control animals, but this result was not statistically significant. *DFX*, Deferoxamine; $CoCl_2$, cobalt chloride.

27% to 30% with the appropriate addition of whole blood to the extracorporeal circuit.

Neurobehavioral Evaluation Scores

After recovery from CPB and DHCA, each animal underwent neurobehavioral evaluations by a blinded observer on postoperative days 1, 2, 4, and 6. Although the neurobehavioral measures indicated a slightly quicker recovery in the deferoxamine and $CoCl_2$ groups (Table E3 and Figure 2), the group-by-time interaction was not statistically significant ($\chi^2 = 53$, $df = 3$, $P = .913$). By postoperative day 6, all animals had recovered to a similar extent, with the most common residual deficits consisting of gait abnormalities or minor ataxia.

Histopathologic Analyses

Histopathologic injury was evaluated in 7 regions of the brain, as indicated above, focusing on acute neuronal injury, reactive gliosis, and frank tissue necrosis. Examination of brain injury by type and region indicated that the thalamus and pons were essentially unaffected (Table E4) and that overall damage was most significant in the hippocampus and cerebellar regions. Only the hippocampus, however, showed a statistically significant effect (Table E5 and Figure 3, *A*). A post-hoc analysis of the group difference on the measure of neuronal loss in the hippocampus demonstrated a significant overall group effect ($\chi^2 = 15.29$, $df = 3$, $P = .002$) and, more specifically, a significant reduction of injury in the deferoxamine ($z = -2.90$, $P = .004$) and $CoCl_2$ ($z = -2.89$, $P = .004$) groups, but not in the hypoxia group ($z = -1.36$, $P = .174$), compared with the control group. Figure E2 is a representative photomicrograph of the diminished hypereosinophilia (representing neuronal loss) in the brain of a $CoCl_2$-treated animal compared with that seen in the brain of a control animal. A similar pattern of protection was seen in the neocortex (Figure 3, *B*) and cerebellum (Figure 3, *C*), but these differences did not reach statistical significance.

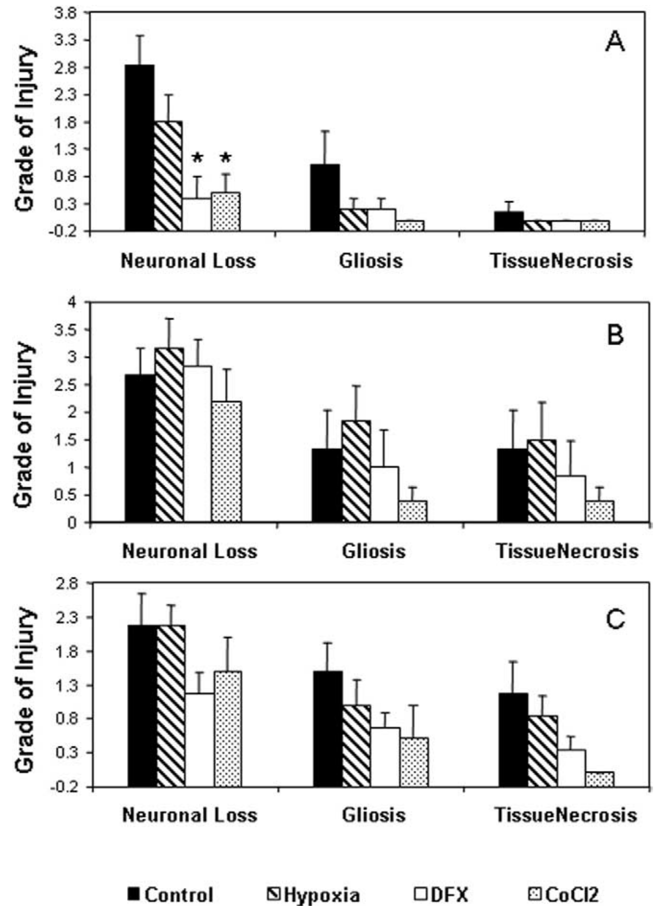

Figure 3. Histopathologic grading of neuronal injury. There was diminished neuronal loss in the hippocampus (A) of animals treated with deferoxamine and cobalt chloride. Similar trends were seen in the frontal lobe (B) and cerebellum (C). *$P < .05$. *DFX*, Deferoxamine; $CoCl_2$, cobalt chloride.

Immunostaining for Cleaved Caspase 3

Finally, as a marker of apoptotic cell death, brain tissue in the hippocampus was immunostained for cleaved caspase 3 to further define the location of the injury because the greatest level of histopathologic protection was seen in this region. The deferoxamine and $CoCl_2$ groups exhibited diminished apoptosis compared with the control group, but this difference was not statistically significant (Table E6 and Figure 4).

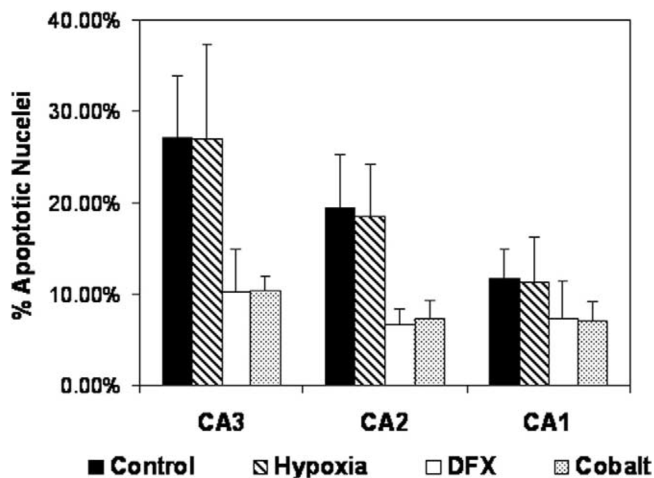

Figure 4. Apoptotic neuronal loss in sectors CA1, CA2, and CA3 of the hippocampus. There was a trend toward protection in the cobalt- and deferoxamine-pretreated groups, but these differences were not statistically significant. *DFX,* Deferoxamine; *$CoCl_2$*, cobalt chloride.

Discussion

Although the cause of brain injury associated with DHCA is multifactorial,[1,11] global ischemia and reperfusion appear to be 2 of the most important factors. Despite the reduction of metabolic demand afforded by deep hypothermia, the relative deficiency of oxygen and glucose during circulatory arrest can cause irreversible cellular damage, particularly with prolonged arrest times. In rat cerebral ischemia models, preconditioning with hypoxia, deferoxamine, and $CoCl_2$ provides an element of neuroprotection by upregulating HIF-1α and its target genes, including EPO,[6,7] vascular endothelial growth factor, and glucose transporter enzymes.[5,11,12] Presumably, these adaptive changes induced by HIF-1α allow more efficient anaerobic metabolism, essentially reducing the metabolic demand during subsequent hypoxia-ischemia.

The current study examined the potential benefits of hypoxic, deferoxamine, or $CoCl_2$ preconditioning on neurological injury in a piglet survival model of DHCA. Although hypoxia did not show a beneficial effect, deferoxamine- and $CoCl_2$-treated animals had significantly diminished histopathologic injury and a trend toward reduced neuronal apoptosis. In addition, animals preconditioned with deferoxamine and $CoCl_2$ had improved neurologic recovery on postoperative day 2 when compared with the control group, although this difference was not statistically significant.

Experimental approaches to neuroprotection in similar models of DHCA have had mixed results. Neuroprotective benefits have been shown with such treatments as thromboxane receptor blockade,[13] intermittent and selective cerebral perfusion,[14,15] allopurinol treatment,[16] systemic steroid pretreatment,[17] and pH-stat blood gas management.[18] Conversely, a number of interventions, such as modified ultrafiltration[19] and pharmacologic cerebroplegia,[20] have not proved to be beneficial. In contrast to the above interventions, which have generally been applied at the time of surgical intervention, cerebral preconditioning would have to be carried out before the operation, a criticism that has precluded preconditioning protocols from being applied clinically to patients with cerebral ischemia as a result of strokes. However, global ischemia associated with DHCA can be anticipated in infants undergoing elective repair of congenital cardiac defects, and therefore preconditioning would be practical in this situation, although the optimal timing of its application remains a matter of further investigation.

Although we obtained positive results with deferoxamine and $CoCl_2$, one difficulty in this study is the fact that hypoxia failed to upregulate HIF-1α or EPO significantly and thus did not have a neuroprotective benefit. Because this was the first attempt to apply hypoxic preconditioning in a porcine model, we attempted to use a protocol similar to what had been described in previous rodent studies (8% oxygen for a period of 3 hours). This level of hypoxia, however, was associated with hemodynamic compromise and death, perhaps related to hypoxic pulmonary vasoconstriction and right-to-left shunting. We therefore exposed the animals to the lowest oxygen content that was tolerable, typically 14% to 16%, providing arterial Po_2 levels in the range of 30 to 40 mm Hg, which might not have been sufficient to stimulate upregulation of HIF-1α and its downstream products. Alternatively, species differences between rodents and piglets could account for the lack of HIF-1α upregulation in response to the 3-hour period of hypoxia that was used in this study. It is therefore possible that an alternative hypoxic stimulus, such as a more prolonged episode of hypoxia, could have induced HIF-1α upregulation and neuroprotection. In addition to longer periods of hypoxia, future investigations should aim to determine whether larger doses of deferoxamine and $CoCl_2$ would have a more pronounced protective effect.

One of the limitations of this study is that although we have shown a correlation between the upregulation of HIF-1α and neuroprotection, we have not conclusively established the mechanism by which this protection occurs. To do so, we would have ideally added further groups combining the preconditioning stimuli with a specific HIF-1α blocker to show that the protective effect would have been abolished. In addition, HIF-1α is known to upregulate more than 20 downstream proteins, many of which could play a role in the neuroprotective process demonstrated. Clearly, further studies designed to delineate the

roles of these other factors are indicated. However, the aim of this study was not to provide new insight into the mechanism of action of HIF-1α but rather to apply the concept of cerebral preconditioning to a clinically relevant animal model. A second limitation is that although we saw differences in upregulation of HIF-1α and EPO after preconditioning with deferoxamine and $CoCl_2$, our study lacked the necessary power to show statistical significance in most cases. The aim of the first phase of the study was to establish the time course of HIF-1α and EPO upregulation so as to optimize the timing of the preconditioning event for the second phase of the study. Therefore it was necessary to euthanize the animals at several time points. Although the small number of animals in each group at each time point resulted in insufficient statistical power to detect even large differences, a properly powered preliminary study would have required an unjustifiably large number of animals. Despite these limitations, the effects of deferoxamine and $CoCl_2$ preconditioning on brain HIF-1α and EPO levels were convincing enough to support further study.

In summary, this study has demonstrated that deferoxamine and $CoCl_2$ preconditioning significantly upregulate brain tissue HIF-1α levels and decrease neuronal injury related to DHCA. This neuroprotective benefit was not seen with hypoxic preconditioning. Until safety, optimal timing, and dosage are determined, this technique is not immediately transferable to the clinical arena but has promising potential as a method to induce ischemic tolerance and prevent neuronal injury in infants undergoing DHCA.

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References

1. Hickey PR. Neurologic sequelae associated with deep hypothermic circulatory arrest. *Ann Thorac Surg.* 1998;65(suppl):S65-70.
2. Clancy RR, McGaurn SA, Wernovsky G, Gaynor JW, Spray TL, Norwood WI, et al. Risk of seizures in survivors of newborn heart surgery using deep hypothermic circulatory arrest. *Pediatrics.* 2003;111:592-601.
3. Semenza GL. Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. *J Clin Invest.* 2000;106:809-12.
4. Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR. Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. *Ann Neurol.* 2000;48:285-96.
5. Jones NM, Bergeron M. Hypoxic preconditioning induces changes in HIF-1 target genes in neonatal rat brain. *J Cereb Blood Flow Metab.* 2001;21:1105-14.
6. Bernaudin M, Nedelec A-S, Divoux D, MacKenzie ET, Petit E, Schumann-Bard P. Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. *J Cereb Blood Flow Metab.* 2002;22:393-403.
7. Prass K, Scharff A, Ruscher K, Lowl D, Muselmann C, Victorov I, et al. Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. *Stroke.* 2003;34:1981-6.
8. Kumar R, Joyner RW, Hartzell HC, Ellingsen D, Rishi F, Eaton DC. Postnatal changes in the G-proteins, cyclic nucleotides and adenylyl cyclase activity in rabbit heart cells. *J Mol Cell Cardiol.* 1994;26:1537-50.
9. Mishra M, Wagner MB, Wang Y, Joyner RW, Kumar R. Expression of cGMP-dependent protein kinase in human atrium. *J Mol Cell Cardiol.* 2001;33:1467-76.
10. Kurth CD, Priestley M, Golden J, McCann J, Raghupathi R. Regional patterns of neuronal death after deep hypothermic circulatory arrest in newborn pigs. *J Thorac Cardiovasc Surg.* 1999;118:1068-77.
11. Murkin JM. Attenuation of neurologic injury during cardiac surgery. *Ann Thorac Surg.* 2001;72(suppl):S1838-44.
12. Prass K, Ruscher K, Karsch M, Isaev N, Megow D, Priller J, et al. Desferrioxamine induces delayed tolerance against cerebral ischemia in vivo and in vitro. *J Cereb Blood Flow Metab.* 2002;22:520-5.
13. Tsui SSL, Kirshbom PM, Davies MJ, Jacobs MT, Kern FH, Gaynor JW, et al. Thromboxane A2-receptor blockade improves cerebral protection for deep hypothermic circulatory arrest. *Eur J Cardiothorac Surg.* 1997;12:228-35.
14. Langley SM, Chai PJ, Miller SE, Mault JR, Jaggers JJ, Tsui SSL, et al. Intermittent perfusion protects the brain during deep hypothermic circulatory arrest. *Ann Thorac Surg.* 1999;68:4-13.
15. Strauch JT, Spielvogel D, Haldenwang PL, Lauten A, Zhang N, Weisz D, et al. Cerebral physiology and outcome after hypothermic circulatory arrest followed by selective cerebral perfusion. *Ann Thorac Surg.* 2003;76:1972-81.
16. Clancy RR, McGaurn SA, Goin JE, Hirtz DG, Norwood WI, Gaynor JW, et al. Allopurinol neurocardiac protection trial in infants undergoing heart surgery using deep hypothermic circulatory arrest. *Pediatrics.* 2001;108:61-70.
17. Shum-Tim D, Tchervenkov CI, Jamal A-M, Nimeh T, Luo C-Y, Chedrawy E, et al. Systemic steroid pretreatment improves cerebral protection after circulatory arrest. *Ann Thorac Surg.* 2001;72:1465-72.
18. Sakamoto T, Zurakowski D, Duebener LF, Hatsuoka S, Lidov HGW, Holmes GL, et al. Combination of alpha-stat strategy and hemodilution exacerbates neurologic injury in a survival piglet model with deep hypothermic circulatory arrest. *Ann Thorac Surg.* 2002;73:180-90.
19. Myung RJ, Kirshbom PM, Petko M, Golden JA, Judkins AR, Ittenbach RF, et al. Modified ultrafiltration may not improve neurologic outcome following deep hypothermic circulatory arrest. *Eur J Cardiothorac Surg.* 2003;24:243-8.
20. Forbess JM, Ibla JC, Lidov HGW, Cioffi MA, Hiramatsu T, Laussen P, et al. University of Wisconsin cerebroplegia in a piglet survival model of circulatory arrest. *Ann Thorac Surg.* 1995;60(suppl):S494-500.

TABLE E1. Neurobehavioral evaluation scores (0 = normal function, 95 = brain death)

Level of consciousness	
Normal	0
Clouded	5
Stuporous	12
Comatose	25
Respiration	
Normal	0
Abnormal	5
Cranial nerves	
Vision absent	1
Light reflex absent R/L	0.5/0.5
Corneal reflex absent R/L	0.5/0.5
Facial sensation absent	1
Auditory absent	1
Gag reflex absent	1
Motor-sensory function	
Flexor response to pain: forelimbs R/L	1/1
Flexor response to pain: hindlimbs R/L	1/1
Righting reflex absent	10
Gait	
Normal	0
Minimal ataxia	5
Moderate ataxia	10
Able to stand	15
Unable to stand	20
No purposeful movement	25
Behavior	
Not drinking	10
Not exploring	10
Total	95

R, Right; *L*, left.

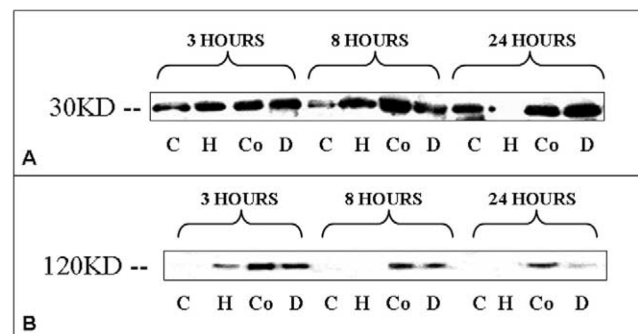

Figure E1. Western blot gels of erythropoietin (A) and hypoxia inducible factor (B) protein, demonstrating more prominent staining in brains of cobalt chloride-treated *(Co)* and deferoxamine-treated *(D)* animals compared with control *(C)* and hypoxia-pretreated *(H)* animals.

TABLE E2. Age, weight, and hemodynamic parameters of piglets undergoing cardiopulmonary bypass and deep hypothermic circulatory arrest

	Control (n = 6)	Hypoxia (n = 6)	DFX (n = 7)	$CoCl_2$ (n = 6)	*P* value
Age (d)	18.8 ± 3.9	21 ± 3.5	19.8 ± 3.7	24.5 ± 3.2	.069
Weight (kg)	5.2 ± 0.9	4.7 ± 0.6	4.7 ± 0.8	5.5 ± 0.8	.249
Prearrest temperature (°C)	16.8 ± 1.2	17.2 ± 1.6	17.3 ± 0.9	17.5 ± 0.5	.881
Prearrest arterial PCO_2 (mm Hg)	47.3 ± 7.4	42.8 ± 5.7	44.6 ± 7.5	46.8 ± 3.1	.508
Mean arterial pressure (mm Hg)					
Before CPB	49.5 ± 3.4	47.5 ± 5.2	51.0 ± 6.2	47.2 ± 2.5	.344
After CPB	59.0 ± 9.6	59.2 ± 8.6	62.5 ± 11.0	57.5 ± 5.2	.736
Hematocrit					
Before CPB	23.2 ± 2.1	25.8 ± 3.8	25.2 ± 3.4	23.0 ± 2.6	.083
On-pump	27.5 ± 1.2	29.7 ± 2.6	29.7 ± 2.9	29.5 ± 2.6	.374
After CPB	28.7 ± 2.9	30.7 ± 2.2	29.3 ± 3.9	29.3 ± 2.2	.675
Cooling time (min)	24.0 ± 1.8	23.3 ± 4.2	23.4 ± 2.1	24.0 ± 2.8	.851
Total CPB time (min)	167 ± 8.4	166 ± 7.9	165 ± 4.7	159 ± 4.1	.173

No significant differences were detected. Results are presented as means ± SD. *DFX*, Deferoxamine; *$CoCl_2$*, cobalt chloride; *CPB*, cardiopulmonary bypass.

TABLE E3. Percentage of animals impaired over time after surgical intervention

	Percentage of animals with neurobehavioral impairment			
	POD 1	POD 2	POD 4	POD 6
Control	100	100	50	33
Hypoxia	100	100	40	40
DFX	100	83	50	33
Cobalt	100	83	33	17

POD, Postoperative day; *DFX*, deferoxamine.

TABLE E4. Percentage of all animals with histologic brain injury by type and region

	Neuronal loss	Gliosis	Tissue necrosis
Frontal lobe	78	48	48
Striatum	96	22	17
Parietal lobe	92	71	67
Hippocampus	64	23	5
Thalamus	8	4	0
Cerebellum	95	64	45
Pons	0	0	0

TABLE E5. Results of Kruskal-Wallis test of group differences in severity of histologic brain injury

	Test of treatment group difference	
	χ^2	*P* value
Over all regions		
Neuronal loss	6.029	.110
Gliosis	3.697	.296
Tissue necrosis	5.399	.145
Over all brain injury types		
Frontal lobe	2.166	.539
Striatum	0.652	.884
Parietal lobe	1.323	.724
Hippocampus	11.491	.009
Cerebellum	5.222	.156

χ^2, χ^2 test with 3 *df*.

TABLE E6. Results of Kruskal-Wallis test of group differences in cleaved caspase 3 among regions of the hippocampus

	Median percentage of apoptotic nuclei				Test of treatment group difference	
	Control	Hypoxia	DFX	Cobalt	χ^2	*P* value
Dentate gyrus	0.32	0.24	0.13	0.26	2.45	.484
CA1	0.11	0.10	0.04	0.07	3.08	.379
CA2	0.15	0.18	0.08	0.07	6.95	.073
CA3	0.21	0.22	0.10	0.11	4.51	.211

DFX, Deferoxamine; χ^2, χ^2 test with 3 *df*.

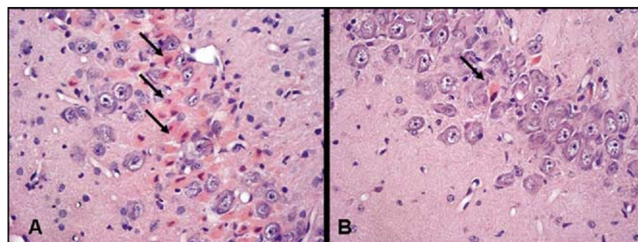

Figure E2. Photomicrographs demonstrating hypereosinophilia *(black arrows)*, which is indicative of acute neuronal loss in the hippocampus of a control animal (A) and a cobalt chloride–treated animal (B).