Cerebral resuscitation in the laboratory: progress and future directions

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Until early 1950’s there did not exist any effective treatment for airway obstruction or cardiac arrest (CA) for laypersons. At the late 50’s there were described isolated steps to establish a patent airway (A), provide mouth-to-mouth breathing (B) and restore circulation (C) with chest compressions. Tying those steps together into an A-B-C sequence became the basis of physiologically effective cardiopulmonary-cerebral resuscitation (CPCR), as the method was called originally.1

Although single steps proved to be effective, the outcomes of out-of-hospital CA treated by CPCR were not encouraging from the very start. The efforts provided by bystanders and medical personnel came often “too little too late”.2 The rapid developments in the field of cardiology in the following decades focused mostly on the circulatory part of resuscitation. Restoration of hemodynamically effective cardiac rhythm became the main criterion for “successful resuscitation”. Cardiology based physicians coined the term “cardio-pulmonary resuscitation” (CPR) and promoted only chest compressions and artificial ventilation. The brain as the key and target organ was somewhat left behind.

In clinical practice, favorable neurologic outcome after CA could be achieved if blood flow is restored within several minutes. However, in the laboratory settings, cerebral neurons can tolerate 1-3 hours of exposure to hypoxia with little histological damage.3 One hour of complete global brain ischemia in monkeys was followed by recovery of many neurons and some of their key functions, e.g. protein synthesis.3 Unfortunately, metabolic recovery does not always result in neurological or functional recovery, as documented in dogs subjected to extended CA that remained in persistent coma despite only scattered neuronal death.4

Interruption of cerebral blood flow (CBF) results in energy failure and electrolyte homeostasis disturbances, resulting in irreversible injury. After restoration of flow, injured neurons can progress to cell death either via necrosis or apoptosis.5 Certain regions of the brain show selective vulnerability to hypoxic-ischemic events. Reperfusion injury is heralded by excessive release of excitatory neurotransmitters, calcium accumulation, protease activation, free oxygen radical formation, and membrane disruption.

Survival and restoration of neurological functions after CA are closely related to the restoration of perfusion. Immediately after restoration of spontaneous circulation (ROSC), CBF is increased to supranormal levels. This “early hyperemia” is followed by a “delayed postischemic hypoperfusion.” Brain metabolism is reduced early after reperfusion. Its association with CBF varies between individual CA models. Dr. Safar postulated the “flow promotion” concept to increase CBF and reduce metabolism with the use of CPB-augmented resuscitation, hypothermia, hemodilution and transient hypertension.6

While ROSC could be claimed in more than half of the CA victims, long-term survival is in the single-digit numbers. In adults, CA is usually caused by an uncoordinated or non-existent cardiac activity, leading to an abrupt cessation of flow. In pediatric population, majority of arrests are of asphyxial origin where the temporal sequence of events is more complex: hypoxemia, hypercarbia, acidosis, hypotension, ultimately resulting in CA. The period of hypoxemic perfusion before CA is probably responsible for greater neurologic damage compared to CA of sudden onset.7

The outcomes of resuscitation of exsanguination CA victims are especially dismal. The situation is complicated by the fact that unlike in normovolemic CA, the missing volume needs to be repleted to enable resuscitation efforts to be effective. However, those victims are especially likely to represent a group of otherwise healthy personnel, “whose hearts and brains are too good to die”. Most of the deaths associated with trauma occur at the site of the accident where medical care is limited. Many of those injuries would be surgically repairable if the victim was treated in the medical care facility. Drs. Safar and
Bellamy suggested to put the victim into a state of “suspended animation” that would buy time for transport, damage-control surgery and delayed resuscitation using CPB.\textsuperscript{8}

The key component of this emergency preservation and resuscitation (EPR) method, as it was recently renamed, is imposing a state of deep hypothermia. Hypothermia is induced by rapid retrograde infusion of a cold solution into the arterial circulation. This allows temporary slowing of metabolic processes. Deep hypothermia has been used in rather preventive than resuscitative fashion with great success in cardiac surgery to create bloodless field and allow to repair both congenital and acquired cardiac diseases.\textsuperscript{9} Initially, a canine model was used to maximize the clinical relevance of the experiments. In the initial phase, a model of extended hemorrhagic shock was used, followed by CPB-assisted cooling. After a period of circulatory arrest with durations from 60-120 min, delayed resuscitation was accomplished with CPB.

Those experiments showed that: a) preceding hemorrhagic shock does not prevent favorable outcome after extended CA when protection is provided by deep hypothermia; b) better results were achieved when deeper levels of hypothermia were used for protection during circulatory arrest, i.e. rather utilizing ultra-profound hypothermia (10 °C) than deep hypothermia (15 °C).\textsuperscript{10}

Two studies explored the effect of various CA durations before the initiation of hypothermia on outcome. Initiation of hypothermia was delayed by 2, 5 or 8 min after the onset of CA. Delays in flush did not change the efficacy of brain cooling. When cooling was delayed by 2 or 5 min, all dogs regained consciousness after resuscitation. In contrast, 8 min delay resulted in poor neurologic outcome. This suggests that the time window for the initiation of hypothermia is limited.\textsuperscript{11}

Extended duration of hemorrhagic shock (~ 2 h to CA) did not prevent favorable survival. Ice-cold flush resulted in deep hypothermia (13 °C), maintained for 60 min, and followed by CPB-assisted resuscitation. While all dogs treated with CPR followed by CPB died < 16 h from multi-organ failure, all but one dog treated with EPR survived to >72 h.\textsuperscript{12}

Surprisingly, to produce intact neurological outcome in this model, it was necessary to extend mild post-resuscitative hypothermia (34 °C) for 36 h -- the group that received post-resuscitation mild hypothermia for only 12 h later deteriorated.

The implementation of post-resuscitative hypothermia was not novel. The use of hypothermia was suggested by Dr. Safar already in his initial “CPCR” diagram.

Laboratory studies in the 1980’s explored the potential of mild hypothermia to protect while limiting complications. Busto et al. found that small increments in intra-ischemic temperatures (33, 34, 36 and 39°C) translate into large differences in neuronal loss in a rat model.\textsuperscript{13} These studies provided evidence that even mild hypothermia could significantly improve outcome in CA.

Timing of hypothermia induction also is critical. Initiating hypothermia during the insult yields the best outcome but is rarely clinically feasible. Delayed hypothermia is beneficial in the early post-insult period but the effect declined over time. Based on studies by Colbourne et al. in gerbils, minimal delay and longer duration are of utmost importance to fully benefit from hypothermia.\textsuperscript{14} De Lange et al. using a rat CPB recently suggested that only the combination of intraoperative cooling, limited rewarming and post-operative hypothermia resulted in amelioration of postoperative cognitive dysfunction.\textsuperscript{15}

The exact mechanism why hypothermia is effective remains to be elucidated. The original concept of hypothermic protection was based on the fact that cerebral metabolic rate is decreased by 5-7% for each degree decrease in body temperature. However, this observation does not explain the ability of even small temperature changes to affect physiology and provide neuroprotection. Protection by hypothermia in experimental central nervous system injury might involve a myriad of mechanisms: maintenance of physiologic ATP concentrations, suppression of glutamate release, attenuation of oxidative or nitratative stress, blunting of the inflammatory response, prevention of energy failure, limitation of cytoskeletal damage, increased levels of neurotrophins, prevention of anoxic depolarization, regulation of gene expression, attenuation of apoptosis or limitation of blood-brain barrier (BBB) injury and vasogenic edema.
The alternate pathway that needed to be explored was the key question if there is any drug that could augment the protective effects of hypothermia. The drugs tested were categorized into one (or more) of the following mechanistic strategies: 1) delaying energy failure, 2) protecting cell membrane integrity, 3) preventing structural degradation, 4) regulating protein synthesis, 5) preventing re-oxygenation injuries, and 6) preserving mitochondria. The goal of this series of experiments was to screen for a breakthrough drug.

Initially, 14 different pharmacological agents were tested in our EPR paradigm using 20 min CA with mild cerebral hypothermia. In controls, saline flush started at 2 min of CA produced survival, but with brain damage. Using 3 to 6 experiments per drug, various doses were flushed into the aortic arch via an intra-aortic balloon catheter, and in some experiments, additional therapy was given during reperfusion with CPB. Remarkably, none of the drugs yielded a breakthrough effect except for the brain penetrating antioxidant tempol.\textsuperscript{16} All dogs that received tempol were normal or near normal, whereas none of the control dogs regained consciousness. However, histological damage was not mitigated by tempol.\textsuperscript{17}

We concluded that the efficacy of drugs paled in comparison to hypothermia. Additional studies performed in a rat model of EPR suggested that the BBB is not disrupted early after EPR, which partially explains the lack of effect of non-brain penetrating drugs.\textsuperscript{18} This is in concert with findings that BBB was not disrupted early after resuscitation in a pediatric asphyxial CA model in rats. CBF after ROSC showed significant regional variations.\textsuperscript{19}

Because of the lack of molecular tools available for dogs, we have recently developed a rat model of EPR that enabled studies of the molecular mechanisms of neuronal and extracerebral injury. Understanding cellular and molecular mechanisms of secondary damage in ischemia-reperfusion injury after CA and the impact of hypothermia and reperfusion on these cascades would allow us both to define specific targets for future interventions and to assess markers of reversibility.

The model is characterized by a favorable survival rate with significant neuronal damage in the hippocampus, associated with microglial activation. Using this model, we will systematically explore the mechanisms of hypothermia and test novel agents.

In the initial studies, we have shown that using intra-arrest deep hypothermia (15 °C) allows to achieve intact outcome, while normothermia was associated with 100% mortality.\textsuperscript{20} The extension of the duration of CA to 60 min still yielded favorable outcome, while further extension to 75 min was associated with increased mortality and neurologic impairment in survivors. Multi-organ failure was probably the underlying cause of death in most rats, with minimal neuronal injury in survivors.\textsuperscript{21} The extended duration of EPR beyond the limit that can yield favorable recovery in rats was associated with increased nitration and ribosylation of selected proteins in selectively vulnerable brain regions.\textsuperscript{22}

Delta-opioid agonist DADLE is an agent known to induce hibernation in summer-active ground squirrels. DADLE was previously reported to increase duration of ischemia in organs harvested for transplantation and improve outcome from experimental CA.\textsuperscript{23} In our model of extended EPR, addition of DADLE during cooling and rewarming did not improve neurologic outcome.\textsuperscript{24} “Hibernation on demand” might be difficult to achieve after the insult, at least with currently available drugs in non-hibernating species.

In more recent studies, we have modified our model to produce neuronal death by imposing a longer duration of normothermic CA before induction of hypothermia, and using higher temperatures. Comparing two different levels of hypothermia (28 and 21 °C) after 20 min CA, we found that functional outcome was significantly improved with deeper levels of hypothermia. Surprisingly, neuronal damage was similar in both groups. In contrast, hypothermia significantly mitigated activation and proliferation of microglia, a hallmark of neuroinflammation. This suggests a novel therapeutic mechanism of hypothermia.

This finding could also provide some explanation to the results of previous studies with rat CPB that showed neurocognitive deficits after normothermic CPB but minimal neuronal death (<2% caspase-3 positive neurons) in selectively vulnerable brain regions.\textsuperscript{23}
Microglial activation has been suggested to be a major cause of delayed neuronal death, most likely through releasing neurotoxic substances, including reactive oxygen radicals, nitric oxide, and pro-inflammatory cytokines. Microglial activation could contribute to neuronal death or microglial-mediated synaptic injury and/or neuronal dysfunction – which could mediate cognitive deficits even in the absence of overt neuronal death. Thus, there may be a specific time window for benefit from inhibition of the early microglial contribution to damage, as well as specific scenario in which blocking microglia could be helpful. It is plausible that microglia effects could be bi-phasic – initially contributing to apoptosis, while later being neuroprotective. Pharmacological modulation of microglial intervention for insults even less than the threshold for neuronal death may help to improve outcome following CA.

Multiple contemporary or novel promising therapies are available to be tested in an effort to find a pharmacological adjunct that would further enhance neuroprotection:

**Minocycline** is a widely used antibiotic with anti-inflammatory and anti-apoptotic properties which has been tested in several models of neurologic injury, including global and focal brain ischemia, traumatic brain injury, spinal cord injury and intracerebral hemorrhage. Most recently, minocycline showed favorable results in a clinical trial in acute stroke patients. The primary effect of minocycline is probably inhibition of activation of microglia. Both motor and neurocognitive behavior were improved by treatment with minocycline, even when the initiation of treatment was delayed for several hours after the insult. Surprisingly, minocycline was also more protective than brief hypothermia after focal cerebral ischemia.

**Clodronate** is a macrophage-depleting agent that does not cross the BBB. Stereotactic administration of clodronate into hippocampus allows us to selectively deplete resident microglia. This newly developed method, by our group, will enable us to study the effects of microglia in temporal sequence.

**Rosiglitazone**, a peroxisome proliferator-activated receptor-gamma (PPAR- ) agonist, have shown neuroprotective effects with acute administration in various CNS injury models. Combination with statins has also shown benefit. Nicotinamide attenuated PARP activation and inflammation, both shown to operate in prolonged CA. Lithium down-regulates pro-apoptotic mechanisms shown to be induced during prolonged deep hypothermic circulatory arrest. Rolipram is a type IV phosphodiesterase inhibitor that increases PKA activation that enhances cell survival pathways and inhibits pro-inflammatory NFKb activation.

The use of hypothermia for protection during CA remains an intriguing area of research with yet many facets to be explored. Even mild hypothermia remains to be a powerful tool that is capable to improve outcome after CA. Compared to the vast unexplored areas of deep and ultraprofound hypothermia, we are yet seeing just the tip of the iceberg.

The rat model has several limitations. Cannulations of tail or femoral arteries result in a retrograde flow. The heart is usually not isolated from systemic circulation and cardiac activity is thus preserved during CPB even under hypothermia. De Lange et al. recently introduced a method of cardioplegia that successfully eliminated this limitation. Also, most studies published so far used young adult rats. While majority of neurocognitive deficits in clinical studies are a direct result of atheromatous embolism, this may not be the optimal model unless additional source of embolic burden is used. In contrast, the use of CPB as a resuscitation tool perfectly mirrors the clinical scenario in which it is planned to be used.

In conclusion, the recent development of a rat model of CPB enables to identify mechanisms underlying neurologic impairment after CPB and/or CA, utilizing techniques that are not available in large animals. Using proteomics and genomics, we will be able to identify the key steps of cell-death pathways and selectively block them. In contrast, expression of neuroprotectant genes that could block apoptotic and perhaps even necrotic cell death can be stimulated to confer additional neuroprotection. The rat CPB model also allows for rapid screening of drugs that could augment cerebral protection, and obviates the cost and labor-intensiveness of large animal models. Neurobehavioral outcome could be easily tested using an established battery of neurocognitive tests for rats, based on the Morris water maze or a modified hole-board test.
The recent advances in the development and optimization of the use of rat CPB now provide us with a sophisticated model that could be used to further enhance our knowledge of post-resuscitation pathophysiology and molecular biology, with direct relevance to cardiac surgery.
