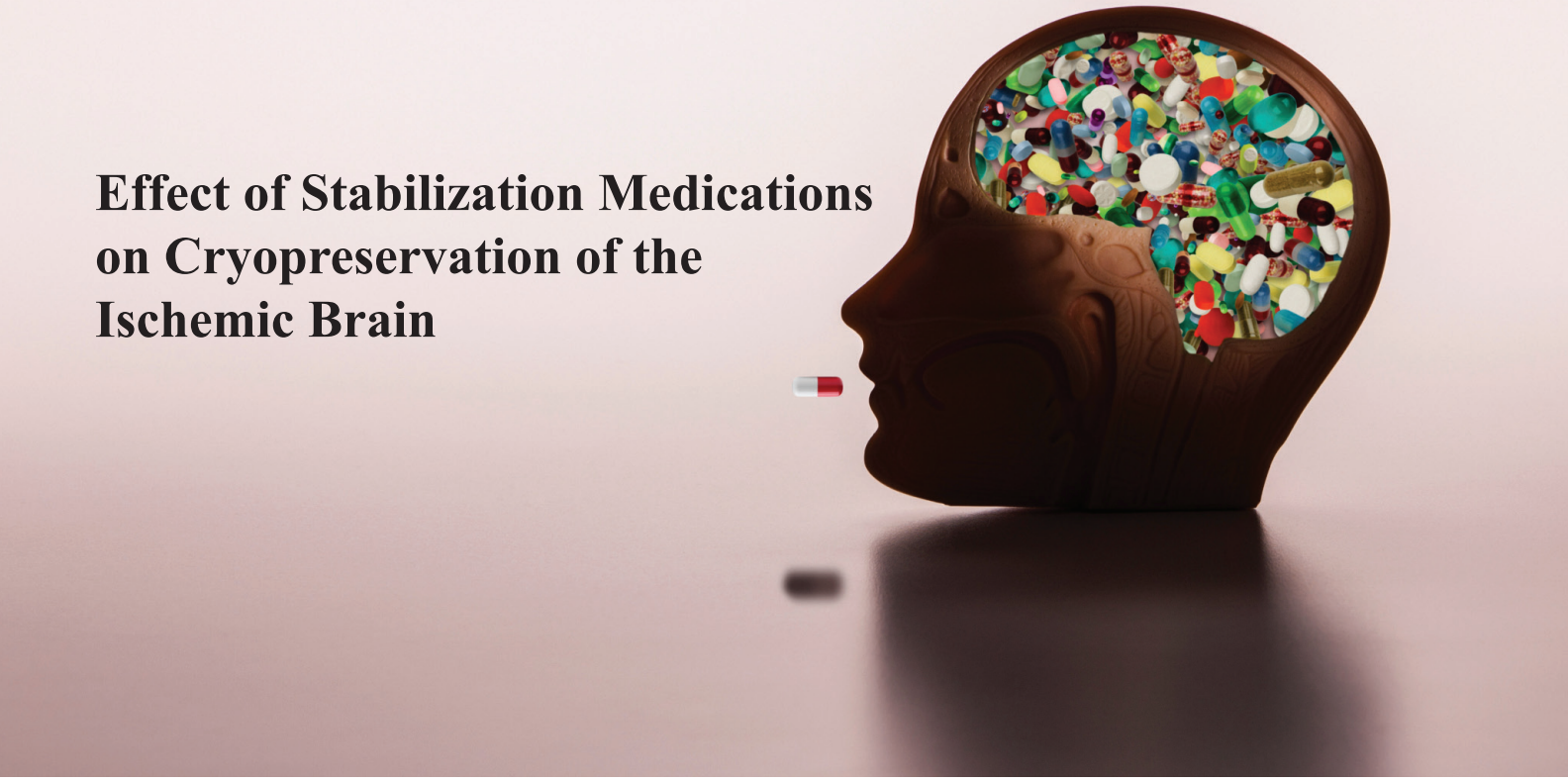
# Effect of Stabilization Medications on Cryopreservation of the Ischemic Brain



## INTRODUCTION

During the period of 2014-2016 Advanced Neural Biosciences, Inc. (ANB) collaborated with the Alcor Life Extension Foundation to investigate the effects of Alcor's stabilization medications protocol on the cryopreservation of the brain. This work builds on prior work at ANB to characterize the effects of ischemia on perfusion and cryopreservation of the brain.

Experimental validation of Alcor's medications protocol is important because: a) these protocols have not been validated using ice formation after cryopreservation of ischemic animals as an endpoint, b) the current number of medications in Alcor's protocol mandates a sensible cost-benefit analysis, and c) improvements to Alcor's medications protocol might be feasible.

To keep the scope of this project reasonable and practical, ice formation after cryoprotective perfusion and cooling to -130° Celsius was used to evaluate the efficacy of the drugs. The guiding hypothesis was that administration of Alcor's transport medications should make an observable difference in terms of ice formation in normothermic and cold ischemic patients. While the limitations of the rat model, and using ice formation as an end-point should be recognized, these experiments can guide further research in large animal models and contribute to a greater understanding of the efficacy of Alcor's stabilization medications protocol.

## BACKGROUND

ANB has conducted experiments into the effects of cerebral ischemia since its inception in 2008 and has reported on its findings in a prior article for this magazine[1]. In short, in our research we established (or further corroborated) that:

- The degree of perfusion impairment and ice formation after cryopreservation is a function of the duration of ischemia.
- Cryoprotective perfusion times increase as the duration of ischemia increases
- Rapid cooling after circulatory arrest reduces perfusion impairment and ice formation after cryopreservation.
- Blood brain barrier breakdown (absence of CPA-induced dehydration) is substantial after 24 hours of cold ischemia and complete after 48 hours of cold ischemia.
- Blood substitution with an organ preservation solution permits ice-free cryopreservation up to at least 48 hours of cold ischemia.
- Composition of the organ preservation matters. The "extracellular" organ preservation solution named MHP-2 produces the best results.
- High perfusion pressure negatively affect outcomes in cryoprotection of the ischemic brain and lower perfusion pressures improve outcome.
- High viscosity cryoprotectants and loading protocols with sharp increases in osmolality improve perfusion of the ischemic brain and reduce ice formation.
- Ischemia-induced whole body edema cannot be mitigated by blood substitution, pharmaceutical treatment, or cryoprotectant carrier solution formulation.
- Blood substitution remains advantageous up to 1 hour of normothermic ischemia.

Our emphasis on normothermic ischemia in our collaboration with Alcor was motivated by two considerations. Firstly, unlike our cold ischemia investigations, we had not been successful in identifying a protocol or treatment that was successful in mitigating the effects of normothermic ischemia. Secondly, a normothermic ischemia model was deemed to be a suitable "test case" to investigate Alcor's stabilization medications because the medications protocol itself were validated in a warm ischemia model.

In our initial studies the medications investigated were administered prior to circulatory arrest. After a comprehensive examination of the effects of Alcor's stabilization medications we broadened our investigations to investigating the effects of administration of stabilization medications *after* circulatory arrest.

## METHODS

Stabilization medications were administered via jugular vein catheters after normothermic ischemia. Cryoprotection of the brain was conducted in-situ using Alcor's open circuit, step-based, field cryoprotection protocol. M22 was introduced through transcardial perfusion and the refractive index was monitored through the jugular veins catheters. Perfusion pressures were limited to 100 mmHg (arterial line pressure). The isolated brain was cooled to -130° Celsius, after which it was inspected for ice formation.

In the experiments were stabilization medications were administered *after* normothermic ischemia medications were administered via jugular vein catheters and were circulated with 3 minutes of chest compressions. In all experiments normothermic ischemia was maintained with a laboratory incubator.

## RESULTS

*Effects of normothermic ischemia on cryoprotectant equilibration*

In our prior cold ischemia research, we established that cryoprotective perfusion times (for a given volume) increase as the duration of ischemia increases. In our current investigations, refractive index samplings showed that larger volumes of cryoprotectant are required to reach the target concentrations in ischemic brains. One important implication of this finding is that cryoprotective perfusion protocols should not be based on perfusing a certain volume of a vitrification solution but to continue perfusion until a target concentration in the brain has been achieved – a practice that both major cryonics organizations practice. In comparison, in our cold ischemia experiments, successful mitigation of perfusion impairment did not require the perfusion of larger volumes of cryoprotectant.

*Effects of anti-thrombotic drugs on cryoprotection of the ischemic brain*

In our prior cold ischemia research, we found that administration of anti-thrombotic drugs (i.e. heparin, citrate, streptokinase, and aspirin) was neither necessary nor sufficient for the mitigation of perfusion impairment and ice formation. In our normothermic ischemia research administration of anti-thrombotic drugs was not necessary for up to at least 1 hour of normothermic ischemia, which indicates that complete inhibition of ice formation in the brain may not necessarily require the administration of anti-thrombotic agents, even after a delay between circulatory arrest and start of stabilization and cryopreservation procedures. As the duration of normothermic ischemia, increases, however, administration of anti-coagulants, was necessary for ice-free cryopreservation of the brain. One caveat to this finding is that in experiments where no medications were administered, drawing samples from the jugular catheters was more challenging (or not possible).

*Effects of citrate on perfusion impairment*

Perhaps the most important finding in our stabilization medications research is that administration of citrate improves perfusion after prolonged periods of normothermic ischemia. While citrate historically has been included in cryonics protocols for cases with long expected transport times, it is only in the last few years that research at our lab and other cryonics-associated labs have established the potency of citrate for mitigating ischemia-induced "no-reflow" in the brain. The combination of citrate and heparin allowed ice-free cryopreservation after 120 minutes of normothermic ischemia at normal perfusion pressure and 160 minutes of low perfusion pressure.

*Effects of perfusion pressure on cryoprotection of the ischemic brain*

In the early literature on the "no-reflow" phenomenon, increasing cerebral perfusion pressure after circulatory arrest was discovered to permit improved recovery. In contract, in cryoprotective perfusion of the ischemic brain after cold ischemia we found detrimental results for increasing perfusion pressure, and improved results when perfusion pressure was lowered. We observed identical beneficial effects for lowering perfusion pressure in cryoprotection of the ischemic brain. When (arterial) perfusion pressure was lowered from 100 mmHg to 80 mmHg ice-free cryopreservation was possible for up to 160 minutes of normothermic ischemia after administration of citrate and heparin.

*Effects of delayed administration of stabilization medications*

In the majority of our experiments, stabilization medications were administered prior to normothermic ischemia. When we changed our protocol to study the effects of delayed administration of stabilization medications (including the combination of citrate and heparin) we did not find benefits in terms of faster perfusion times, reduced whole body edema, or prevention of BBB breakdown, after administration of drugs after 30 minutes of normothermic ischemia. After 15 minutes, however, administration of stabilization medications (most notably, Alcor's "abbreviated protocol") reduced perfusion times compared to no administration of stabilization medications.

*Effects of cerebral ischemia on the blood brain barrier*

One of the most pronounced, and (presumed) adverse effects, of contemporary brain cryopreservation technologies is that vitrification solutions induce substantial dehydration of the brain. This phenomenon is not observed in cryopreservation of the ischemic brain.

In our experiments with cold ischemia we observed progressive breakdown of the BBB as the duration of cold ischemia increases. In the rat model, normothermic ischemia produces rapid breakdown of the BBB and no cryoprotectant-induced dehydration could be observed after 15 minutes of normothermic ischemia. None of the medications or protocols tested was able to mitigate this ischemia-induced compromise of the BBB.

*Effects of stabilization medications on whole body edema*

One of the most daunting problems in human cryopreservation concerns the effect of ischemia on vessel permeability. As the duration of cold and/or normothermic ischemia increases, so does the degree of whole body (and cerebral) edema during cryoprotective perfusion. In our research we have not find evidence that either the administration of stabilization medications or the composition of organ preservation solutions or cryoprotectants can mitigate this kind of edema. After 15 minutes of normothermic ischemia, weight loss is still routinely observed after completion of cryoprotective perfusion but this phenomenon is seem both with and without administration of stabilization medications.

## DISCUSSION

Choice of animal model and sample size should prompt caution about the results we report but in conjunction with theoretical predictions, similar findings in the experimental literature and other laboratories, this research can at least guide other researchers and medical practitioners in their decision making.

All our efforts to understand the effects of cerebral ischemia on cryopreservation of the brain strongly corroborate that the so called "no reflow" phenomenon also pertains to cryoprotective perfusion. A number of caveats are in order. To our, surprise ice-free cryopreservation was still possible up to at least 1 hour of normothermic ischemia, regardless of medications administration. This result raises important questions about the role of anti-thrombotic agents in human cryopreservation.

Further research into the question why delivery of larger perfusate volumes (relative to controls) seemingly overcomes the "no-reflow" phenomenon is an interesting research topic. It is conceivable that the hyper-osmolality of modern vitrification solutions recruit edematous fluids back in the circulatory system and that this mitigates perfusion impairment. Another explanation is that BBB breakdown secondary to cerebral ischemia prevents ice-free cryopreservation through dehydration and thus requires more perfusate volume and longer perfusion times to render the brain resistant to ice formation. Preliminary research in our laboratory with protocols that do not dehydrate the brain support the latter interpretation.

Stabilization medications administration was found to shorten perfusion time if administered at15 minutes of circulatory arrest and were found to be necessary to eliminate ice formation when the period of normothermic ischemia was longer than 1 hour. The efficacy of citrate has been particular notable. Our research cannot answer whether the effectiveness of citrate reflects its properties as an anti-coagulant specifically, or as a calcium chelator in general. Impaired calcium regulation has been identified as one of the main drivers of the ischemic cascade in the brain. Our finding that the combination of citrate and heparin is more potent than citrate alone may indicate a role for aggressive anti-thrombotic interventions in cryopreservation of the ischemic brain (at least beyond one hour of normothermic ischemia).

It was somewhat concerning, and unexpected, that we did not observe any benefits for any other medications in Alcor's extensive stabilization protocol on variables such as ice formation, weight gain, perfusion time, or BBB integrity. The only exception to this observation was administration of Alcor's abbreviated protocol after 15 minutes of ischemia. It is interesting to note that this duration of ischemia is approximately the same as the duration of ischemia that allowed for successful cerebral resuscitation in the research that underpins Alcor's stabilization medications protocol. Pre-administration of citrate and heparin permitted ice-free cryopreservation up to 150 minutes of normothermic ischemia but whether there is a sound rationale for administration of these medications (or Alcor's comprehensive protocol) *after* 30 minutes of normothermic ischemia needs further theoretical and experimental study.

Can whole body edema be prevented during cryoprotective perfusion? Modification of the vitrification solution might be advantageous in cases without ischemia but neither our cold ischemia research nor our normothermic ischemia research has been able to found protocols that mitigate ischemia-induced whole body edema. Whether vascular leaking of this nature can be addressed through different pharmaceutical agents or novel perfusion protocols remains to be seen but this challenge remains the most formidable to date.

Independent validation of our findings in different animal models and using larger sample size is recommended. Perhaps the most significant modification of our research model would be to implement direct visualization of (brain) vessels to obtain a more empirical understanding of the nature of "no-reflow," the development of edema, and the mechanism of cryoprotectant equilibration in the ischemic brain. ■

## REFERENCES

1. "Advanced Neural Biosciences", *Cryonics* Magazine, April 2015