Experimental Validation of EBF Cryopreservation Protocol After Normothermic Ischemia

Introduction

Human biostasis requires the use of a vitrification solution to eliminate ice formation during cryopreservation. The current generation of vitrification agents are informed by the use of high concentrations of cryoprotectants to prevent freezing and optimization of the carrier solution to improve viability and reduce chilling injury. One current non-proprietary agent in the field is a solution named VM-1, which was developed by the late cryobiologist Yuri Pichugin for the Cryonics Institute. As a low-cost, non-proprietary, agent, VM-1 remains the ideal candidate to investigate as a vitrification solution for EBF. In our research we focused on two solution composition and perfusion protocol properties:

(1) Optimization for perfusion of the ischemic brain.
(2) Optimization for field cryoprotection and dry ice transport.

This research built on the histological characterization of VM-1 cryoprotected brains that was conducted in 2017-2018 for the Hirsch Foundation (https://hirsch-foundation.org/en/project/histological-characterization-of-vm-1-cryoprotected-brains). In this research it was demonstrated that under ideal conditions, cryoprotection of the brain with VM-1 preserved the fine structure of the brain, although in a shrunken state.

It has been recognized that normothermic- and cold ischemia can lead a number of phenomena that interfere with good cryoprotectant perfusion, such as blood coagulation, red cell aggregation, cellular and cerebral edema, vessel obstruction etc. As of writing, Advanced Neural Biosciences is the only cryobiology company that had conducted comprehensive research into the mechanisms of ischemia-induced cryoprotectant perfusion impairment and its resolution.

So far, the discoveries and progress made has been limited to formal recommendations how to optimize pre-perfusion stabilization protocols (such as the prioritization of rapid sodium citrate and heparin administration). This research aimed to identify perfusate composition and perfusion protocol changes that might mitigate ischemia-induced perfusion impairment, including high dose streptokinase administration, pulsatile perfusion, and decompressive craniectomy. We chose a 3-hour postmortem interval based on the observations that Tomorrow Biostasis may frequently find itself responding to cases that have such postmortem delays due to regulatory reasons.

Experiments

The experimental setup and methodologies for this research are broadly similar to those used for the 2017-2018 Hirsch Foundation research.

We conducted a series of (non-ischemic) VM-1 control experiments, a series of VM-1 experiments under ischemic conditions (i.e., 3 hours of normothermic ischemia) and altered standard protocol / equipment / perfusate composition to improve upon the data observed in the ischemic conditions.

Metrics to compare the data between controls, normal VM-1, and alternative protocols / solutions included: the increase of venous index values over time, total perfusion time, and comparison of pre- and post-perfusion weight (which is a strong indicator of ischemia), and post-cryopreservation ice formation.

The following experiments were conducted (n=5). All experiment used 70% w/v VM-1 unless otherwise noted. Experiments in orange were added in addition to the contracted experiments to further explore potentially effective protocols.

- Control VM-1
- Control (pulsatile pump)
- Control (80% w/v VM-1)
- Ischemia (3 hours normothermia)
- Ischemia + M22
- Ischemia + Post-mortem streptokinase (2,500,000 IU)
- Ischemia + Post-mortem sodium nitrite (1%)
- Ischemia + Pulsatile perfusion
- Ischemia + Oxygenation during cryoprotectant perfusion
- Ischemia + Decompressive craniotomy during cryoprotectant perfusion
- Ischemia + VM-1 in ANB-1 carrier solution
- Ischemia + 65% w/v VM-1 / 3% PVP K12 / 2% Z-1000 / 1% X-1000
- Ischemia + Post-mortem streptokinase + sodium nitrate (combined)
- Ischemia + Hypertonic VM-1 (2x m-RPS concentration)
- Ischemia + 80% VM-1 w/v
- Ischemia + 80% VM-1 w/v (4 hours perfusion of 80% concentration at 80 mmHg)

Methods

Animals were euthanized by introduction of isoflurane gas anesthesia (SomnoSuite Low-Flow Anesthesia System, Kent Scientific) and place in an incubator at normal body temperature. Animals were then packed in ice and cooled to 5°C. A thoracotomy was performed to give open access to the heart. Perfusions were started at 5°C and performed via transcardial perfusion, in which an 18-g cannula is introduced to the heart through the apex of the left ventricle and secured in the ascending aorta. Solutions were perfused through the vasculature by means of a peristaltic pump and allowed to flow out of the animal through a nick in the right atrium of the heart. The abdominal aorta was clamped to prioritize perfusion of the head. Perfusion pressure in the arterial line was not allowed to exceed 100 mmHg. Temperature of the animal (i.e., the brain) was kept at 5°C or lower. A rectal temperature probe was used to measure the animal's temperature and another thermocouple was used to measure the arterial line temperature (perfusate temperature).
All cryoprotectant solutions were kept at ≤5°C prior to introduction. Additives such as streptokinase were added to the m-RPS-2) washout solution.

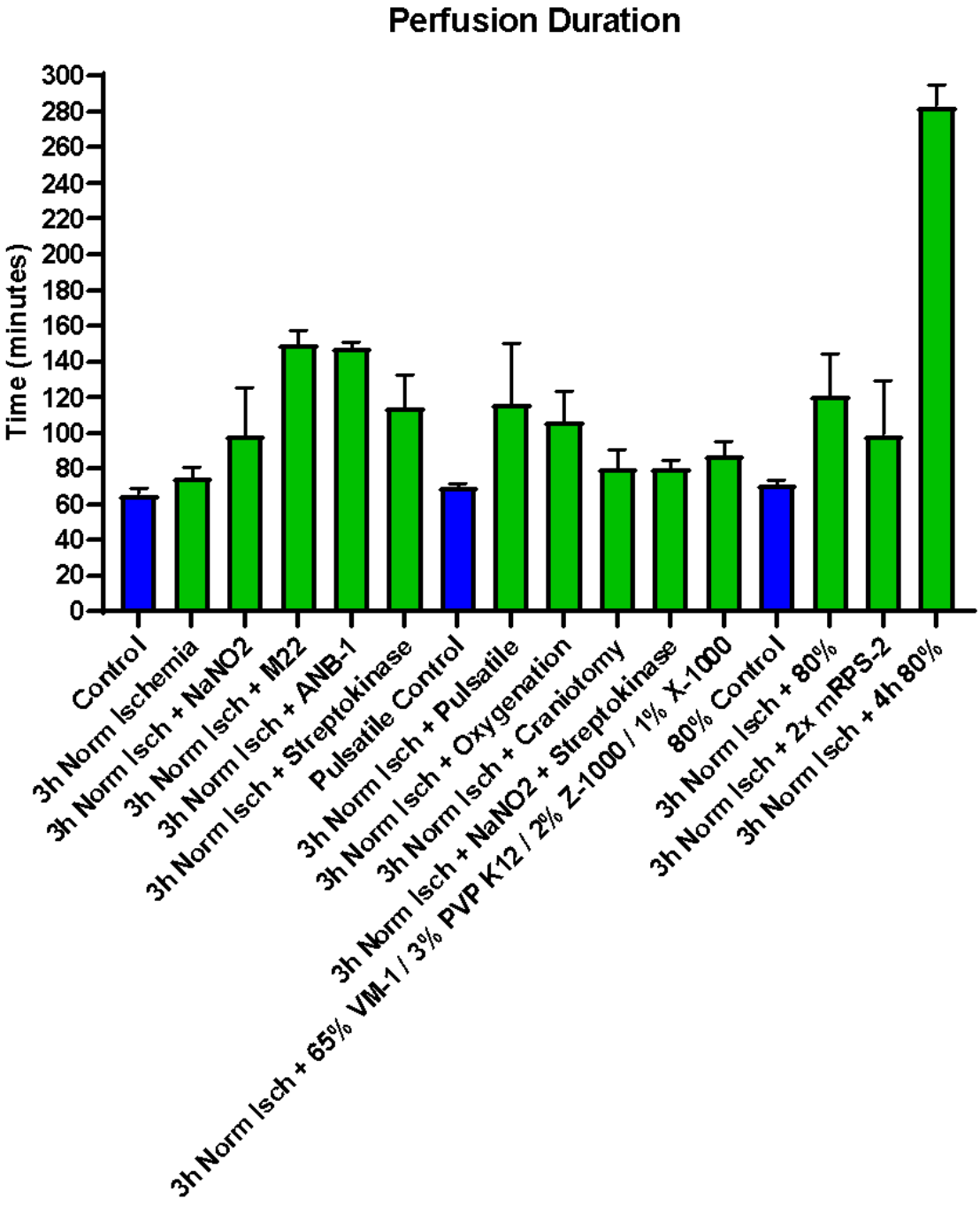
1. mRPS-2 (100 ml) at 0°C - 5°C
2. 5% w/v (100 ml) at 0°C - 5°C
3. 10% w/v VM-1 (100 ml) at 0°C - 5°C
4. 30% w/v VM-1 (100 ml) at 0°C - 5°C
5. 70% w/v VM-1 at 0°C - 5°C

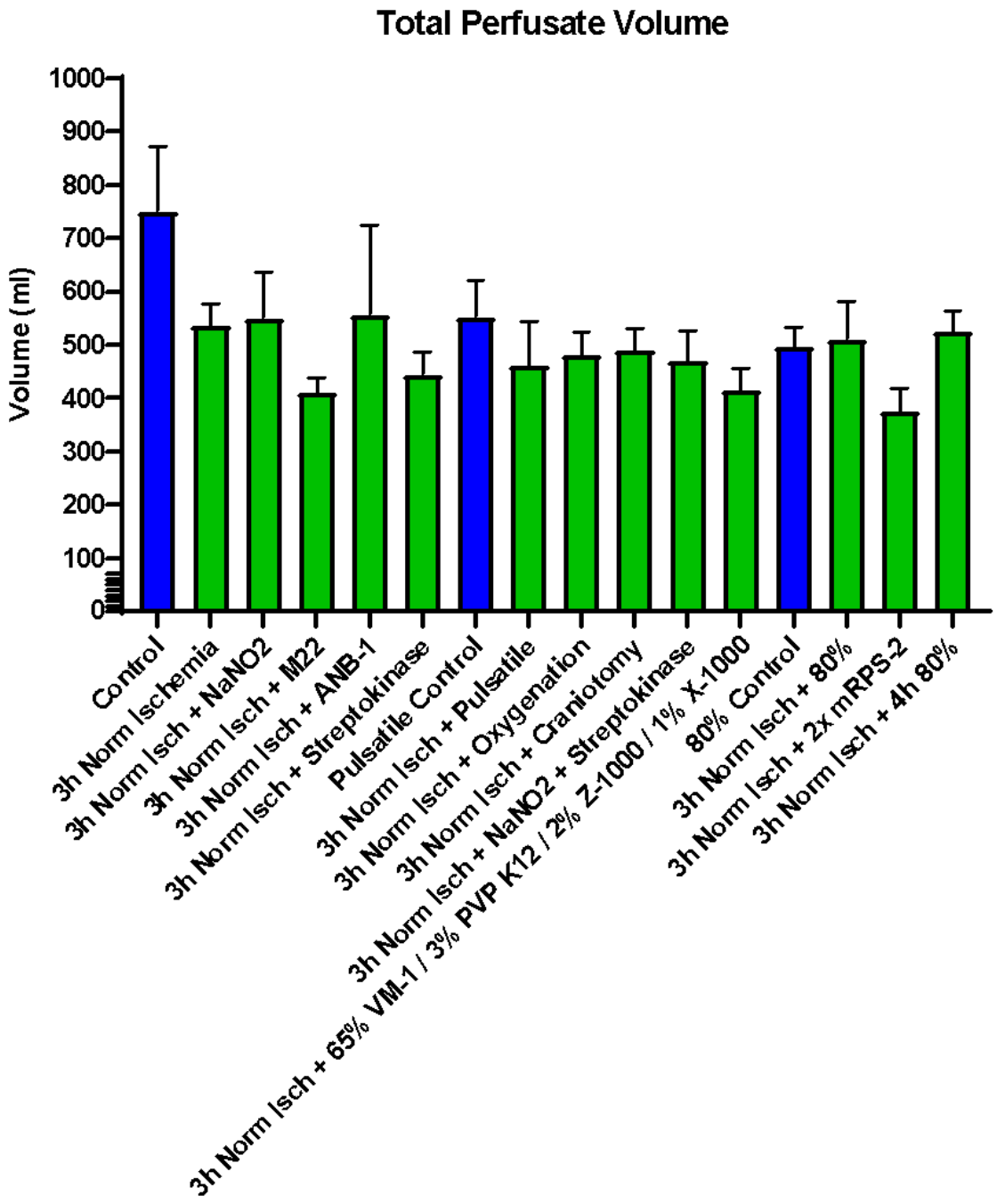
Cryoprotective perfusion was completed when the venous effluent refractive index measured the terminal concentration necessary for vitrification for VM-1 (or alternative solution) or when the last step was perfused for 1 hour, whichever comes *last*.

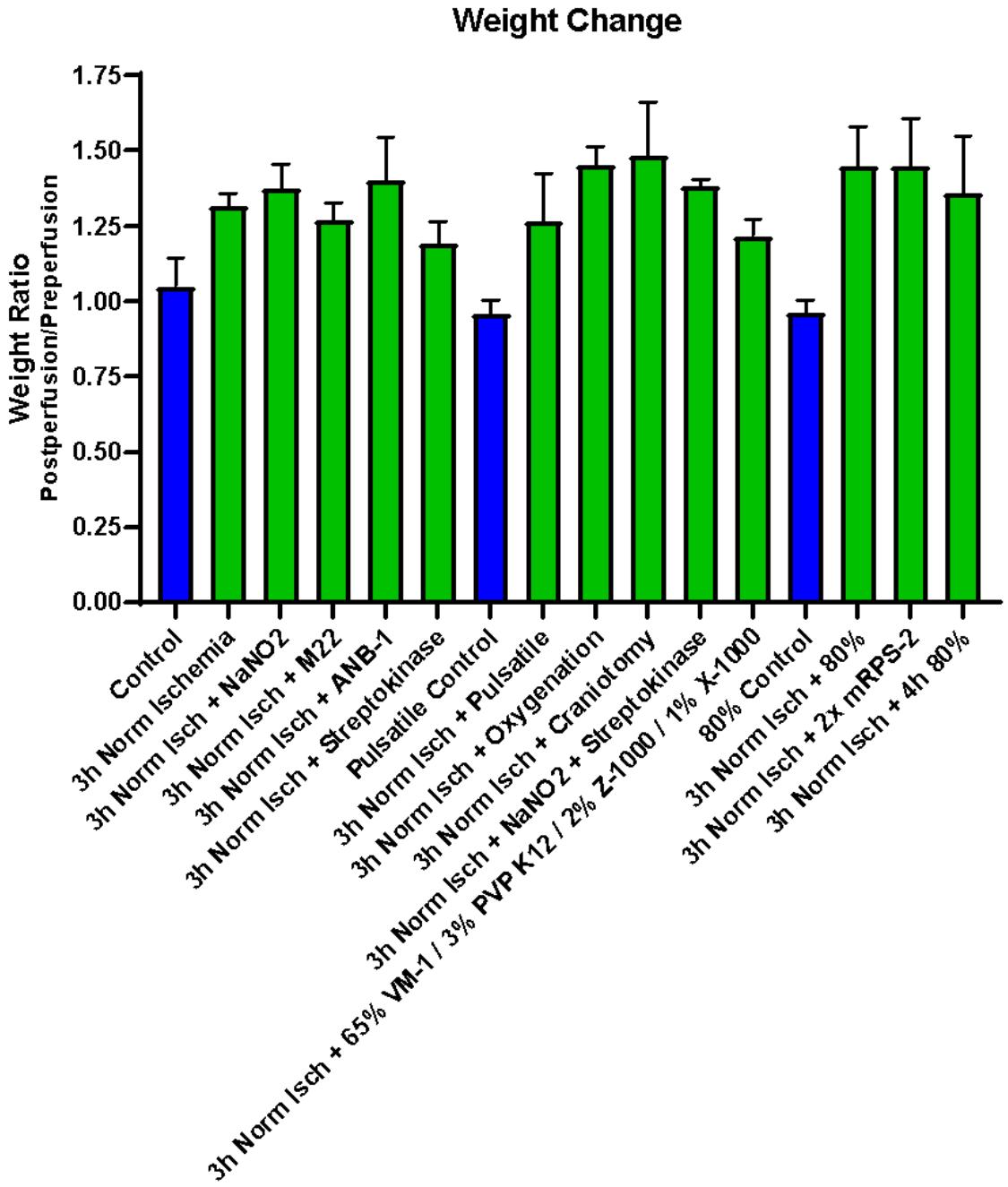
After completion of cryoprotective perfusion the brain was isolated, photographed to determine shrinking, and cooled to - 130°C and inspected for ice formation.

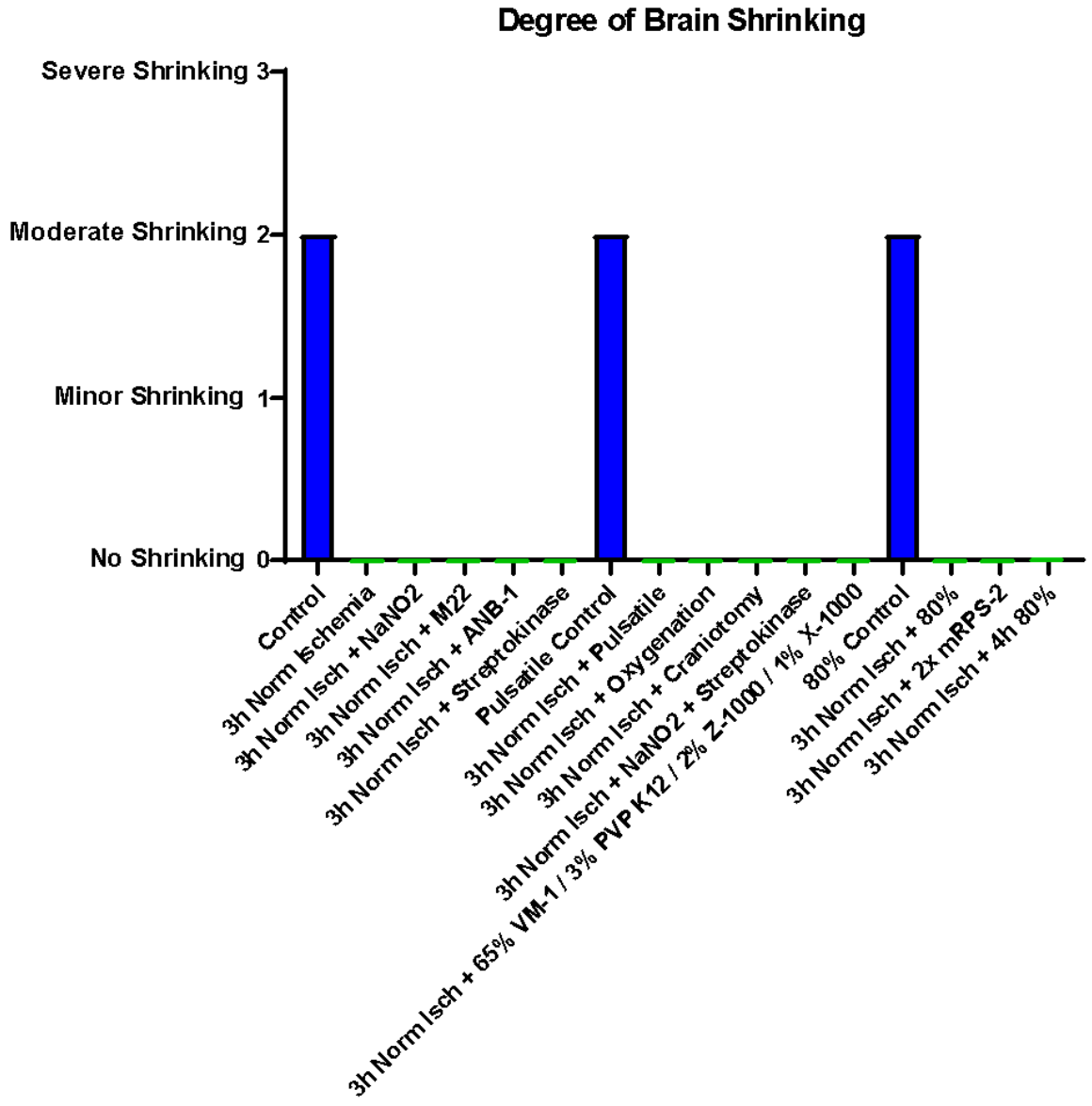
Results

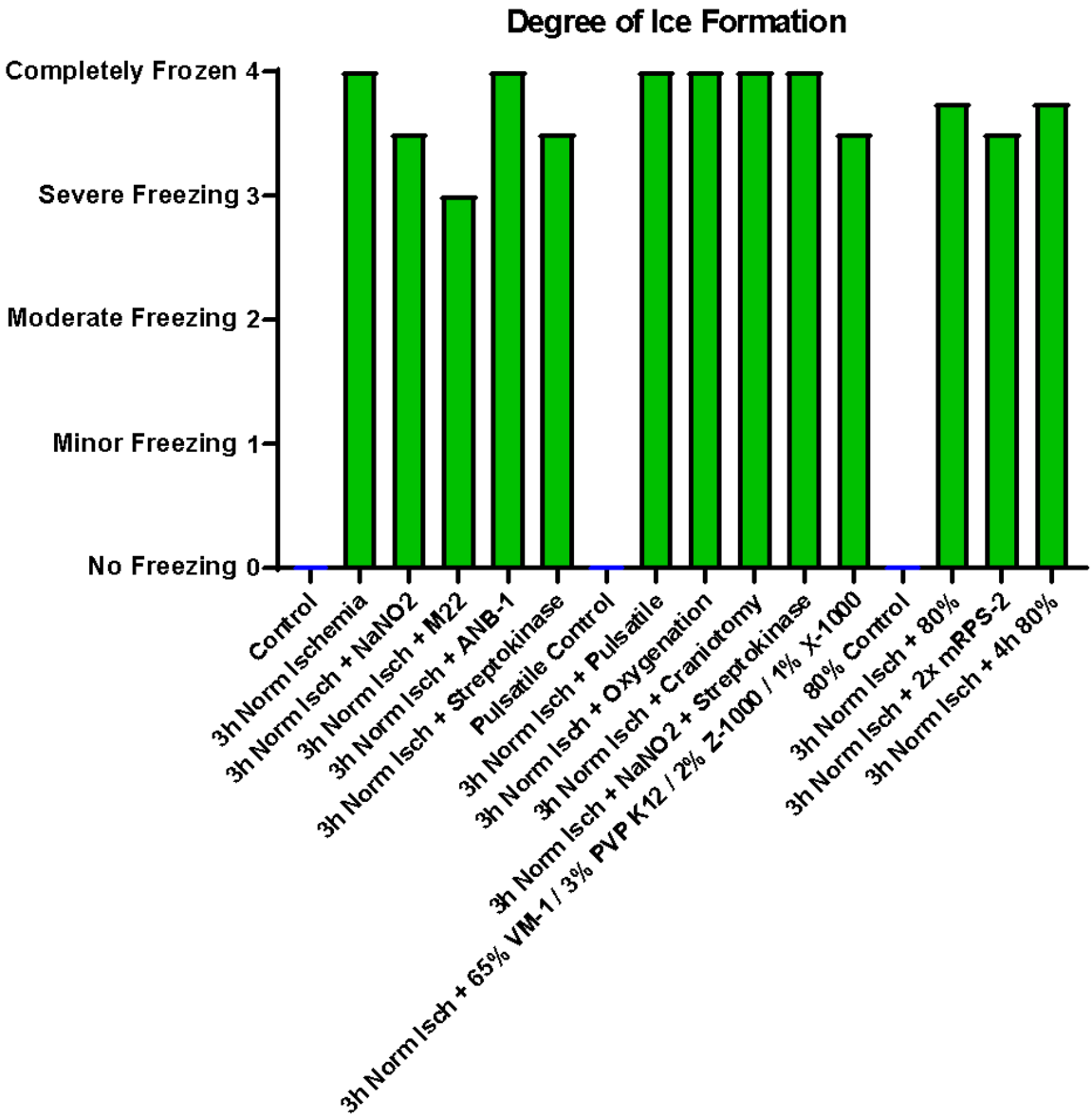
All experiments were conducted as n=5 and results are displayed as averages or median (for degree of brain shrinking and degree of ice formation).




Total Perfusate Volume
Volume (ml)
1000
900
800
700
600
500
400
300
200
100
0
Control
3h Norm Ischemia
3h Norm Isch + NaNO2
3h Norm Isch + M22
3h Norm Isch + ANB-1
3h Norm Isch + Streptokinase
Pulsatile Control
3h Norm Isch + Pulsatile
3h Norm Isch + Oxygenation
3h Norm Isch + Craniotomy
3h Norm Isch + NaNO2 + Streptokinase
3h Norm Isch + 65% VM-1 / 3% PVP K12 / 2% Z-1000 / 1% X-1000
80% Control
3h Norm Isch + 80%
3h Norm Isch + 2x mRPS-2
3h Norm Isch + 4h 80%


Weight Change
Weight Ratio
Postperfusion/Preperfusion
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Control
3h Norm Ischemia
3h Norm Isch + NaNO2
3h Norm Isch + M22
3h Norm Isch + ANB-1
3h Norm Isch + Streptokinase
Pulsatile Control
3h Norm Isch + Pulsatile
3h Norm Isch + Oxygenation
3h Norm Isch + Craniotomy
3h Norm Isch + NaNO2 + Streptokinase
3h Norm Isch + 65% VM-1 / 3% PVP K12 / 2% Z-1000 / 1% X-1000
80% Control
3h Norm Isch + 80%
3h Norm Isch + 2x mRPS-2
3h Norm Isch + 4h 80%


Degree of Brain Shrinking
Severe Shrinking 3
Moderate Shrinking 2
Minor Shrinking 1
No Shrinking 0
Control
3h Norm Ischemia
3h Norm Isch + NaNO2
3h Norm Isch + M22
3h Norm Isch + ANB-1
3h Norm Isch + Streptokinase
Pulsatile Control
3h Norm Isch + Pulsatile
3h Norm Isch + Oxygenation
3h Norm Isch + Craniotomy
3h Norm Isch + NaNO2 + Streptokinase
3h Norm Isch + 65% VM-1 / 3% PVP K12 / 2% Z-1000 / 1% X-1000
80% Control
3h Norm Isch + 80%
3h Norm Isch + 2x mRPS-2
3h Norm Isch + 4h 80%


Degree of Ice Formation
Completely Frozen 4
Severe Freezing 3
Moderate Freezing 2
Minor Freezing 1
No Freezing 0
Control
3h Norm Ischemia
3h Norm Isch + NaNO2
3h Norm Isch + M22
3h Norm Isch + ANB-1
3h Norm Isch + Streptokinase
Pulsatile Control
3h Norm Isch + Pulsatile
3h Norm Isch + Oxygenation
3h Norm Isch + Craniotomy
3h Norm Isch + NaNO2 + Streptokinase
3h Norm Isch + 65% VM-1 / 3% PVP K12 / 2% Z-1000 / 1% X-1000
80% Control
3h Norm Isch + 80%
3h Norm Isch + 2x mRPS-2
3h Norm Isch + 4h 80%

Discussion

Since its inception, ANB has made optimization of cryoprotective perfusion one of its main research topics. In our cold ischemia research, we established the importance of low perfusion pressures for perfusion of the ischemic brain (between 60 mmHg and 100 mmHg), adding streptokinase to the initial washout step, and the importance of blood substitution with an "intra-cellular" hypothermic organ preservation such as MHP-2 prior to transport.

In our "pre-medication" normothermic research we established that a combination of sodium citrate and heparin can extend the period after which good perfusion is still possible after normothermic ischemia, to at least 2 hours post-mortem, provided that these drugs are administered prior to the start of the ischemic period. When sodium citrate and heparin are administered *after* circulatory arrest, these benefits decline between 15 and 30 minutes of circulatory arrest.

The most formidable challenge in cryoprotection of the ischemic brain is to improve outcomes after prolonged periods of normothermic ischemia without pre-medication. In this research we chose a duration of 3 hours of normothermic ischemia to screen a comprehensive set of perfusate and perfusion protocol changes to improve perfusion and ice formation outcomes.

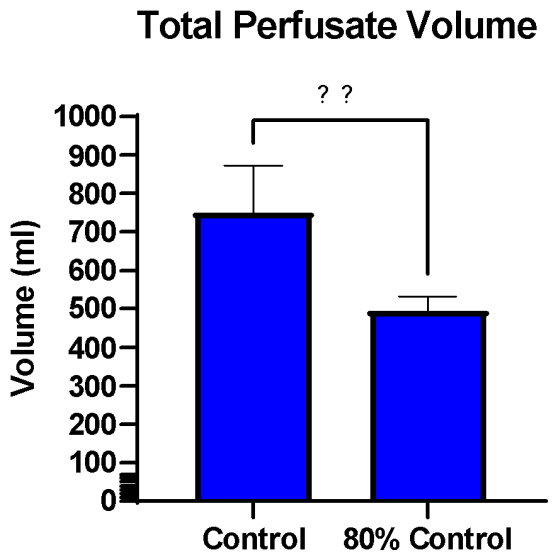
Our interventions included anti-thrombotic interventions (high-dose streptokinase administration), dilation of blood vessels (sodium nitrate administration), alternative cryoprotectants (M22), alternative carrier solutions (ANB-1), oxygenation of the perfusate, the use of pulsatile pumps, higher concentrations of CPA (80% w/v VM-1), increasing carrier solution tonicity (M22, ANB-1, 2x m-RPS-2), and removal of parts of the skull (decompressive craniotomy).

As can be readily seen in the ice formation median results, none of our interventions substantially reduced the amount of ice formation in the brain after cooling to -130 degrees Celsius. To our knowledge, some of these experimental methods have never been investigated in experimental whole-brain neural cryobiology research (pulsatile flow, ultra-high concentration CPA's). In particular, given observations of ischemia-induced brain swelling in experimental research and CT scans, the use of decompressive craniotomy was expected to be a particularly potent mitigation strategy.

The difference between all experimental protocols covered the range of "severe freezing" and "complete freezing" and does not seem to yield clear protocols that would expect to significantly "move the needle" for cryoprotection of the ischemic brain, at least not for 3 hours of normothermic ischemia or longer. It is interesting to note that 3 out of 4 experimental results that look slightly better involved cryoprotectants or carrier solutions that were mildly or strongly "hypertonic" (M22, 2-x m-RPS-2, and addition of PVP K12 and X-1000 and Z-1000 to VM-1). While hypertonicity has been hypothesized to mitigate "chilling injury", its role in blood substitution and cryoprotection in human cryopreservation warrants more exploration.

For pulsative flow and the 80% w/v VM-1 experiments we also conducted controls to understand how these protocols compare to regular 70% w/v VM-1 protocol. These results could offer potential new research directions, regardless of the perfusion impairment mitigation properties of these solutions. For example, perfusate volume utilization was significantly lower for using 80%

w/v VM-1 to get to a 75% w/v venous refractive index than for 70% w/v to get to a 65% w/v venous refractive.



The table below shows that using 80% w/v VM-1 yields a venous refractive index reading equivalent of 65% w/v VM-1 substantially faster than using a 70% w/v VM-1 solution.

Perfusion time to 65% w/v venous refractive index

	Control	Pulsatile control	80% control
00:05	1.37854	1.37716	1.37684
00:10	1.40528	1.38536	1.40674
00:15	1.40226	1.39604	1.40958
00:20	1.4059	1.4058	1.41436
00:25	1.41264	1.40924	1.41778
00:30	1.41648	1.4096	1.42066
00:35	1.41662	1.40904	1.42138
00:40	1.4154	1.41168	1.42352
00:45	1.41568	1.41534	1.42528
00:50	1.4162	1.41382	1.42702
00:55	1.41822	1.41308	1.42814
00:60	1.41718	1.41285	1.42848

A solution of 80% w/v VM-1 can be classified as a true “equilibrium” vitrification solution. Equilibrium vitrification solutions do not require a critical cooling- or warming rate to prevent ice formation and can even be held for prolonged periods at a single subzero temperature without freezing. Given the faster equilibration of these solutions to a safe CNV (Concentration Necessary to Vitrify), and their stronger resistance to ice formation when perfused to higher

concentrations (70% w/v to 75% w/v range) further exploration of true equilibrium solutions for the use of human cryopreservation, and “field cryoprotection” in particular, is recommended.

Our research has not produced clear benefits for any of the alternative experimental protocols investigated, but we suggest to further explore these protocols for other durations of normothermic ischemia (i.e., cold ischemia models or shorter periods of normothermic ischemia). We also recommend further research into the potential benefits of transitioning to hypertonic, equilibrium-type vitrification solutions.

References

Perfusion & Diffusion in Cryonics Protocol
By Benjamin Best
https://www.benbest.com/cryonics/protocol.html

A detailed technical primer to understand the use of perfusion technologies in cryonics.

Human Cryopreservation Research at Advanced Neural Biosciences (2011)

by Aschwin de Wolf and Chana Phaedra
http://immortalistsociety.org/anb_research.htm

This article documents the earliest experimental research of *Advanced Neural Biosciences* concerning the effects of ischemia on the cryopreservation of the brain.

Effect of Stabilization Medications on Cryopreservation of the Ischemic Brain (2017)

By Aschwin de Wolf
https://www.biostasis.com/pdfs/medications.pdf

Comprehensive study to explore the effects of medication administration on the cryopreservation of the ischemic brain.

An Introduction to Field Cryoprotection (2014)

By Aschwin de Wolf
https://www.alcor.org/library/field-cryoprotection/

First technical exposition of the rationale and technical aspects of “field cryoprotection.”