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SUPPLEMENTARY INFORMATION

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Restoration of brain circulation and cellular functions hours post-mortem

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Supplementary Discussion

Previous studies have reported *in cranio* perfusion of dog and macaque brains using allogenic whole-blood[48,49], as well as the isolation[50] and perfusion[14,51,52] of the small rodent (i.e., guinea pig) brain utilizing oxygenated cerebrospinal fluid. However, these earlier studies were conducted under hypothermic conditions on acute brain tissue preparations with negligible or shorter PMI's, in contrast to the present study. To the best of our knowledge, this is the first report describing the use of a synthetic, hemoglobin-based, cytoprotective perfusate and extracorporeal perfusion system to restore and maintain microcirculation as well as molecular and cellular functions in the large mammalian brain under normothermic *ex vivo* conditions following an extended PMI.

Pioneering studies have sought to ascertain the maximal duration of global cerebral ischemia after which the capacity for functional recovery is lost[40,42,43,53]. Interestingly, there were significant discrepancies between tolerated normothermic ischemia times, ranging from 4 minutes to 1 h[17,40,42,43,53,54]. These heterogeneous observations suggest that brain survival is not dependent on ischemia time *per se*, but is instead related to variations in the recovery process (i.e., extent of post-anoxic/ischemic reperfusion, aggregation of blood particles, etc). Therefore, it may be possible that with the appropriate interventional protocol, the large mammalian brain could withstand longer periods of circulatory arrest. Indeed, our findings indicate that oxygen consumption rates were comparable with previously reported *in vivo* values[55], but glucose consumption was higher than normal[56] yet in line with rates reported for brains recovering from prolonged global ischemia[57]. This mismatch could be due to replenishing brain glucose stores or fueling anaerobic mechanisms. Yet, since previous work has shown that younger animals demonstrate a greater resilience to anoxic injury than adults[58], it is important to note that we used brains from young animals (i.e., 6-8 months of age). Therefore, the increased plasticity in younger-aged brains may have contributed to the overall cellular recovery.

As has been extensively reviewed, tissue damage after global, or focal, ischemia results from the convergence of pathophysiological processes such as loss of ionic homeostasis, excitotoxicity, inflammation, and oxidative stress, leading to cell death[2,10,59,60]. Hence, we reasoned that the most appropriate approach would include a combination of cytoprotective agents and technical innovations that circumvents many obstacles associated with *in vivo* reperfusion or *ex vivo* perfusion with whole blood, including immune-mediated tissue damage, blood cell obstruction, and microemboli formation. Moreover, the technology described herein affords greater latitude for intervention with real-time manipulation of gas and electrolyte concentrations, temperature dynamics (i.e. rate of rewarming), and drug administration without the constraints of systemic side-effects in a model organism with a brain composition similar to humans.

With its gyrated neocortex, readily-accessible vasculature, and complex morphology, the porcine brain represents an appropriate experimental model for studying brain responses to global anoxia as it more closely resembles the human brain than the smaller, lissencephalic brain of rodents, which are commonly studied mammalian species. Indeed, one of the most notable differences between pig and rodent brains is the difference in gray-to-white matter ratio, with the rodent brain comprising only 14% white matter, while pig and human approximate 30 and 50%,

respectively[61,62]. This particular distinction influences not only neurovascular architecture and function, but also neural connectivity, metabolism, and physiology, which play critical roles in anoxic/ischemic injury[63,64].

In light of this, we developed a perfusate formulation that addresses independent mechanisms that affect both gray and white matter to diminish cell injury and death. In conjunction, we also designed the perfusate as an echogenic, hypertonic, and acellular solution containing an anti-coagulative hemoglobin-based oxygen carrier in order to prevent edema formation, ensure proper delivery of nutritive and protective agents throughout the cerebral microvasculature, and allow ease of detecting perfusion status via ultrasonography, which distinguishes the B*Ex* perfusate from previously reported organ preservation solutions[65-69]. Indeed, the acellularity of the B*Ex* perfusate may have allowed ubiquitous microvascular reperfusion by circumventing many of the thrombotic, vascular, perivascular, and/or hemolytic mechanisms previously reported to cause perfusion deficits[70-72]. While we cannot at this time exclude the possible development of delayed cellular death previously reported to start 72 h to weeks after ischemia[73,74], we have also incorporated both anti-apoptotic and anti-necroptotic agents within the perfusate to halt the progression of these delayed mechanisms. In doing so, we observe a decrease in cell death in brains subjected to B*Ex* conditions, in comparison to control- or 10 h PMI brains.

These compounds, as well as neuronal activity blockers, are washed out during the preparation of acute hippocampal slices for electrophysiological recordings, possibly explaining the observed discrepancy between whole cell patch-clamp recordings and global cortical activity. Further studies are necessary to determine whether the brain is able to regain global cortical activity following 4 h of global anoxia/ischemia with the current experimental set up (i.e. anoxic/ischemic times, length of perfusion, and/or perfusate formulation). With prolonged perfusion studies, we should be able to obtain a better understanding of how cells within the brain respond to extended time-points. It is remotely conceivable that as the technology develops, the brain may regain the ability to emit global (i.e., ECoG/EEG) signals. However, in the event that this were to occur, we were ready to swiftly implement countermeasures, including, but not limited to, reducing the temperature of the brain in order to diminish metabolic activity, and/or administering general anesthetic agents (which were already at-hand during the perfusion experiments) to maintain an isoelectric ECoG reading.

This immediate response to a potential positive ECoG reading follows from our position that we are not aiming to maintain awareness in the *ex vivo* brain, if it were to be re-established. Given the possibilities for inadvertent suffering posed by the restoration and maintenance of remnant awareness, any experimentation aimed at purposefully maintaining a brain with global electrical activity, without the aforementioned interventions to diminish such activity, should be subject to more extensive ethical review. It is our firm belief that this platform can have broad use in addressing various neuroscientific questions that are independent of global electrical activity. Indeed, more liberal and productive use of this platform is likely to be made if standard operating procedures are in place to address the ethical questions associated with re-establishing and maintaining global network activity.

Overall, these findings indicate that a combinatorial approach to addressing anoxic/ischemic injury is capable with restoring and maintaining certain molecular and cellular functions in the brain even hours post-mortem. Utilizing this system, investigators can begin addressing long sought-after questions pertaining to the large mammalian brain, such as conducting mesoscale tracings of long-range connections, while also performing novel pharmacological penetrance and kinetic studies.

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