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Unilateral hypoxic brain perfusion for generation of cerebral ischemic injury in rat.

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Introduction

Stroke is the third leading cause of death in the United States, and among the leading causes of disability affecting 795,000 Americans each year [1]. Statistics show the mean lifetime cost per person to be \$140,080, totaling approximately \$53.6 billion per year. The leading risk factors include elevated systolic blood pressure, hypertension, diabetes mellitus, hypercholesterolemia, and current smoking [2, 3]. Other non-modifiable factors attribute to the risk [1, 2]. Overall, the prevalence of these risk factors in the population places stroke as a serious healthcare problem, and a substantial economic burden to the U.S. and those affected. Ischemic stroke is one of the two main stroke classifications, representing 87% of strokes seen clinically. Ischemic cerebral infarcts arise from occlusion or stenosis in extracranial, intracranial, and small cerebral arteries, which can be mechanistically initiated by thrombosis or by embolic events [4, 5, 6]

Numerous experimental animal models exist which reproduce the neurological deficits resulting from cerebral ischemia [7, 8]. These models/protocols provide the basis to trial neuroprotective and neuroregenerative therapies. Although larger animal species have traditionally been used, rodent models, namely rats, provide the platform for such experiments. Rodent-based experiments are more cost-effective, and despite smaller size, produce favorable results similar to larger species due to similarities in cerebral vascular anatomy. The majority of the ischemic models can be categorized as embolic or hypoperfusion/hemodynamic methods. Other models mimic more hemorrhagic stroke. However, hypoperfusion methods seem to be the most commonly employed technique to induce cerebral ischemia in rodents.

The earliest of these methods was the four vessel occlusion model which induced cerebral ischemia by bilateral common carotid artery (CCA) occlusion with bilateral vertebral artery electrocoagulation. Although this model produced favorable results, experiments were difficult to reproduce in other labs due to demanding technical requirements of the surgery to complete the procedure. Later, a more reproducible two vessel occlusion model was developed implementing bilateral CCA occlusion under systemic hypotension. The histopathological

changes in both models were similar, showing prominent bilateral lesions to the hippocampus, striatum, and frontal cortex [8,9]. One of the variants of the ischemic methods was the Levine preparation, a straight forward attempt to produce unilateral cerebral ischemia based on unilateral CCA occlusion and progressive exposure to anoxic conditions. The peculiarity of this approach was that ischemia was performed under normotension which avoided some of the complications that could arise from systemic hypotension. Histopathological evaluation of these experiments showed lesions in areas supplied by the middle cerebral artery (MCA) and included the frontal cortex, striatum and hippocampus [10].

The goal of the present study was to establish a new experimental protocol/model of unilateral cerebral ischemia for the development of a cost-effective and highly reproducible hemispheric neurological deficit in rat. Traditionally, as described above, such models employing unilateral common carotid artery occlusion under hypotensive conditions have been unsuccessful in developing consistent histologically-defined neurological pathology due to significant collateral cerebral blood flow. We speculate that unilateral cerebral ischemia can be achieved by the intra-carotid artery infusion of isothermic buffered solution while performed under normotensive conditions. Following the determination of optimal infusion time to reach sufficient cerebral ischemia that overcomes the ischemic penumbra and avoids animal death, we predict a neurological/histopathological deficit that includes 1) contralateral hemiparesis, and 2) ipsilateral histologic lesions of the grey and white matter to areas supplied by the middle cerebral and anterior cerebral arteries. In regards to the latter, we expect to see damage to the ischemic-sensitive grey matter of the hippocampus, in addition to the cerebral cortex and striatum.

Materials and Methods

All procedures were approved by the University of California, San Diego Animal Care Committee. 20 Male Sprague-Dawley rats (250-275 g) were single-housed in standard cages with corncob bedding. Animals had access to food and water *ad libitum*, and a 12 h light/dark cycle was used through out the study.

Induction of cerebral ischemia

All procedures were performed under aseptic conditions. Animals were anesthetized with 4% isoflurane in an oxygen-air mixture (1:1), and maintained with 1.5-2.0% isoflurane delivered by face mask. Body temperature was measured using a rectal thermometer. Core body temperature was maintained using a hydrothermo heating blanket. Arterial blood pressure was monitored by tail artery catheter. The right common carotid artery was cannulated using a polyethylene 10 (PE 10) tubing for infusion access [Figure 1]. The anoxic infusate consisted of isotonic heparinized (1:250) phosphate buffer saline (pH 7.4), and was infused via an automated syringe infuser (Harvard Apparatus Model 975) fitted with a 50 cc syringe, delivered at 3.0-3.3 cc/min (the reported carotid artery flow rate). The temperature of the anoxic solution was maintained

throughout infusion at 37°C using a controlled water bath at the same temperature. For blood collection, the right femoral artery was cannulated using 22 gauge needle and connected to a second heating coil system maintained at 37°C. This system was connected to a 60 mL syringe suspended at 54 cm above the animal to maintain a systemic mean arterial pressure (MAP) of 40 mmHg. Animals were divided into three groups to simulate different ischemic episodes using anoxic infusion for 2, 4 and 6 minutes (n=5 each). 5 naïve rats served as controls.

Following anoxic infusion, blood was manually re-infused over 15 min, and 1 mg protamine sulfate was administered intraperitoneally to reverse heparin anticoagulation effect. Arterial blood pressure was monitored for 20 minutes to allow stabilization. Arterial lines were then removed, incisions were closed with 4-0 non-absorbable sutures, and animals were allowed to recover.

Post-operative analysis

2-21 days after cerebral ischemia, animals were assessed for the development of contralateral motor deficit using a modified neurological scoring system developed by Garcia et al [Table 1]. Briefly, animals were assessed using a 0-3 scale (0= no movement, 3=normal) on: 1) ability to ambulate in their cages, 2) symmetry of movements (all four limbs), and, 3) symmetry of forelimbs (outstretching while held by tail) [11].

Perfusion Fixation and Histological analysis

For histological analysis, all rats were anesthetized with pentobarbital (100 mg/kg) and phenytoin (25 mg/kg) administered intraperitoneally, and transcardially perfused with 200 mL of heparinized saline followed by 250 mL of 4% paraformaldehyde (PFA) in phosphate buffer (pH=7.4). Animals were decapitated, and whole heads (skin, muscle, brain left intact) were post-fixed in 4% PFA overnight (approximately 8-10 hrs) at 4°C. Following post-fixation, brains were completely dissected and cryoprotected in 30% sucrose phosphate buffer for 3-10 days at 4°C. To facilitate left and right orientation, brains were dissected along the interhemispheric fissure. Using a Leica CM1900 cryostat, 30 µm coronal sections were cut from bregma -2.0 mm to -6.0 mm to specifically include the hippocampus. Free floating sections were mounted to microscopy slides and underwent histological staining according to Fluoro-Jade immunohistological protocol to investigate presence of apoptotic neurons in the ipsilateral hippocampus.

Statistical analysis

Analysis of physiological data was to be carried out by one-way ANOVA for multiple comparisons followed by Dunnett post hoc test. For analysis of neurological outcome and histopathology non-parametric tests were used. For each individual study (i.e. ischemic time), tests for overall main effect are performed with the Kruskal-Wallis test. Significant main effects ($p < 0.05$) are probed further through comparisons of each test condition using the comparison of experimental Mann-

Whitney test (unpaired two group test). To correct for the increased probability of type I error with multiple comparisons our significance level for post-hoc Mann-Whitney test comparisons was set at $p=0.01$. Data are expressed as mean \pm SD.

Results

Neurologic Evaluation

Animals were assessed for neurologic dysfunction using a modified score system developed by Garcia et al [11] [Table 1]. Rats in each timing cohort showed no evidence of expected contralateral deficits from immediate post-infusion recovery and following observation 2 to 21 days post-infusion, achieving neurological scores of 6 (data not shown). All rats survived the infusion procedure, and none died during the recovery or observational period. Four rats did developed ptosis and myosis ipsilateral to CCA cannulation that was independent of infusion time. These symptoms spontaneously resolved 7 days post-procedure.

Histological analysis

Processed brains were stained to identify neuronal necrosis within the hippocampus. No innate Fluoro-Jade immunofluorescence was seen in control animals. Animals that underwent 2 and 4 minute infusion also had absence of staining. 2 out of 5 rats after 6 minute infusion showed hippocampal staining although to a minimal degree [Figure 2 D,E]. No Fluoro-Jade staining was seen on the contralateral hippocampus in any animal in this study.

Discussion

Reproducible unilateral brain ischemia via anoxic solution infusion was not achieved in this study. All rats showed complete neurologic function following infusion. Expected neurological dysfunction would include contralateral upper motor neuron weakness, hyperreflexia, and likely hypertonia, consistent with middle cerebral artery or anterior cerebral artery ischemia within the anterior cerebral circulation. The transient post-infusion ptosis and miosis observed in several animals does not represent an expected deficit predicted from this ischemic territory. These findings were likely secondary to damage of sympathetic fibers originating from the superior cervical ganglion during dissection of the carotid sheath to isolate the CCA.

Despite the lack of neurological sequelae from infusion, slight hippocampal Fluoro-Jade staining was observed. Fluoro-Jade is an anionic acidic dye with innate immunofluorescence at 488 nm wavelength, and has been used extensively to study neuronal degeneration with a predominate focus in the ischemic-sensitive hippocampus. [12, 13]. The precise mechanism of Fluoro-Jade is unknown, however is postulated to be due to its affinity for basic molecules expressed or exposed in the degenerating neuron [14]. It is believed to have a similar molecular basis to silver impregnation staining [15]. The presence of neuronal necrosis as indicated by Fluoro-Jade suggests that ipsilateral

ischemia was achieved at 6 minutes infusion, although not sufficient to surpass the neuronal threshold to overcome the ischemic penumbra.

There are numerous animal models that attempt to recreate cerebral ischemia, but despite the utility of small animal models, inconsistencies from animal to animal have been documented [16]. Such variability may lead to differences in the degree of ischemia which may not represent the expected pathological outcome. Ischemic and post-ischemic temperature has been one parameter leading to inconsistency outcomes. Indeed, one of the most difficult parameters to control in this study was intra-ischemia animal temperature where a range of 33.6 °C to 37.8 °C was observed. Mild hypothermia to 34 °C has been shown to have considerable neuronal protection during ischemic induction via modified four-vessel occlusion [16, 17, 18, 19, 20]. As much as 60% of hemispheres in animals undergoing ischemia showed no evidence of hippocampal necrosis studied by hematoxylin and eosin staining. A similar percentage of preservation was also observed in the central and dorsolateral striatum [16]. Another study showed animals undergoing ischemia when exposed to moderate hypothermia at 30°C had hippocampal neuron cell counts similar to sham animals [17]. Additionally, a decrease in as little as 2 °C has been shown to produce significant neural protection in gerbils [19]. These studies were limited to histological or energy metabolite changes, and the investigation for motor or sensory dysfunction was not conducted. However, Green et al. demonstrated hippocampal neuron sparing in animals undergoing intra-ischemic hypothermia and no significant impairment in learning set tasks such as submerged platform finding compared to normothermic ischemic animals [18].

Previously, Busto et al. concluded that rectal temperature poorly and unreliably reflected brain temperature during ischemia; temperatures above 33 °C were needed to achieve consistent pathology [16]. A more sophisticated subcutaneous or intracranial temperature feedback heating system was needed. Given that temperature was controlled using a simple water heating blanket system set 37° C, a significant discrepancy between core body temperature and brain temperature must have likely existed. Continuing on the topic of temperature, another contributor to hypothermia may have resided within the anoxic infusion solution which was heated to 37 °C via a water heating coil system. Despite careful attention to minimize the distance between the heating coil system and the CCA, it is possible that appreciable infusate cooling occurred in transit to systemic circulation.

Anatomical variations within rat cerebral vasculature have been documented in literature and the possibility of ischemic protection from abundant collateral anastomoses is logical [21, 22, 23]. However such protection in the present experiment is unlikely. Oliff et al. showed significant rat strain and vendor difference in collateral blood flow to MCA territories however reported no significance to number, luminal diameter, or MCA-ACA density to lesion sizes following MCA occlusion [22]. In the present experiment, controlled infusion through the CCA would have reproduced a physiological flow rate that would have prevented perfusion via contralateral collaterals.

Post-ischemic inflammation has been implicated in early tissue damage. Since only Fluor-Jade immunohistochemistry was used to assay for pathology the degree of inflammation secondary to ischemia is unknown. Future study should include reactive microglial inflammatory markers such as IBA-1. Similarly, hematoxylin and eosin staining via light microscopy can show subtle histological changes as cells undergo necrosis that are not shown through other methods, and should be included to give further clues if ischemia was present.

In conclusion, unilateral hypoxic ischemia via common carotid artery infusion was not achieved. Despite evidence of hippocampal necrosis as indicated by Fluoro-Jade immunohistochemistry, animals displayed no qualitative neurological deficits. It is likely that intra-ischemic systemic and intracranial hypothermia created a neuroprotective environment during infusion. Further studies will necessitate sufficient temperature control based on intracranial temperatures.

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