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Article

Keywords:

Posted Date: February 13th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3920465/v1

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Additional Declarations: No competing interests reported.
Enhanced Brain Connectivity and Hemodynamic Stability in Canine Stroke Models Treated with Flow Augmentation Therapies: A Resting-State fMRI Study

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ABSTRACT

The acute phase of ischemic stroke presents a critical window for therapeutic intervention, where novel approaches such as hyper-acute cerebral flow augmentation offer promising avenues for neuroprotection. In this study, we investigated the effects of two such therapies, NEH (a combination of norepinephrine and hydralazine) and Sanguinate (pegylated bovine carboxyhemoglobin), on resting-state functional connectivity, global mean signal (GMS), and blood oxygen level-dependent (BOLD) time lag in a pre-clinical canine model of stroke via permanent occlusion of the middle cerebral artery (total of n = 40 IACUC-approved mongrel canines randomly split into control/natural history and two treatment groups). Utilizing group independent component analysis (ICA), we identified and examined the integrity of sensorimotor and visual networks both pre- and post-occlusion, across treatment and control groups. Our results demonstrated that while the control group exhibited significant disruptions in these networks following stroke, the treatment groups showed remarkable preservation of network integrity. Voxel-wise functional connectivity analysis revealed less pronounced alterations in the treatment groups, suggesting maintained neural connections. Notably, the treatments stabilized GMS, with only minimal reductions observed post-occlusion compared to significant decreases in the control group. Furthermore, BOLD time-lag unity plots indicated that NEH and Sanguinate maintained consistent hemodynamic response timing, as evidenced by tighter clustering around the line of unity, suggesting a potential neuroprotective effect. These findings were underscored by robust statistical analyses, including paired T-tests and Mann-Whitney U tests, which confirmed the significance of the connectivity changes observed. The correlation of BOLD time-lag variations with functional outcomes highlighted the clinical relevance of these neuroimaging parameters in evaluating the impact of stroke and the efficacy of therapeutic interventions. Our study supports the inclusion of NEH and Sanguinate in stroke treatment protocols and suggests their potential to extend the therapeutic window and improve patient outcomes.

Introduction

Ischemic stroke, a rapid-onset neurological event characterized by the sudden loss of blood circulation to an area of the brain, results in a corresponding loss of neurological function. This condition, primarily caused by an embolism or occlusion of an artery, stands as a leading cause of morbidity and mortality worldwide [1]. The hyper-acute phase, particularly the first 8 hours following the start of a stroke, is deemed critical for therapeutic intervention. During this period, the penumbra, the brain tissue surrounding the occlusion and at risk yet still potentially salvageable, underscores the importance of early intervention [6,7].

The standard of care in stroke management traditionally involves thrombolyis, such as the administration of tissue Plasminogen Activator (tPA), and mechanical thrombectomy to restore blood flow [8,11,13]. However, these methods have limitations, including a narrow therapeutic window and potential hemorrhagic risks. In this context, flow augmentation strategies, including NEH (a combination of norepinephrine and hydralazine) and Sanguinate (pegylated bovine carboxyhemoglobin), have emerged as novel therapeutic approaches during this hyper-acute phase [9,10,13,21,22]. These interventions aim to enhance cerebral blood flow to ischemic regions, potentially preserving brain tissue and function, and could complement existing thrombolytic and thrombectomy procedures by extending the treatment window and mitigating associated risks [13,17,18,40].

In assessing the effectiveness of these flow augmentation therapies, key neuroimaging metrics such as functional connectivity, global mean signal (GMS), and BOLD (Blood Oxygen Level Dependent) time delay are crucial [6-8, 23-33]. Functional connectivity, referring to the temporal correlation between spatially remote neurophysiological events, indicates functional integration between separate brain regions [12,15,23]. The GMS, indicative of the average signal across the entire brain, reveals insights into the global state of brain function [10]. Additionally, the BOLD time delay, a critical aspect of functional MRI
(fMRI), measures the lag between neural activity and the subsequent hemodynamic response, providing a window into the efficiency and health of neural processing [11].

This study, utilizing a pre-clinical canine model, aims to investigate how hyper-acute cerebral flow augmentation influences these neuroimaging parameters in the context of ischemic stroke. We hypothesize that NEH and Sanguinate can preserve functional connectivity, maintain GMS, and minimize BOLD time delay during the critical hyper-acute phase, thereby offering neuroprotective benefits. Our research contributes to the stroke treatment field by potentially paving the way for the inclusion of these novel interventions in standard stroke treatment protocols.

**Results**

**Group Independent Component Analysis (ICA)**

**Sensorimotor Network Insights from Group ICA:** Figure 1a showcases the sensorimotor networks identified through group ICA, conducted on all subjects both pre- and post-occlusion. This network was also distinctly observed when group ICA was exclusively performed on pre-occlusion subjects, suggesting its integral presence regardless of the stroke event. A subsequent comparison, achieved by subtracting the pre-occlusion network results from the all-subjects results, revealed the persistence of these networks in the post-occlusion phase. The post-occlusion networks, depicted in blue, are overlaid on Johnson et al.’s 2020 T1 weighted canine atlas, offering a comprehensive anatomical context.

**Visual Network Dynamics and Comparative Analysis:** Similarly, Figure 1b elucidates the visual networks, following the same analytical methodology as applied to the sensorimotor networks. These networks were found in both the all-subjects (pre and post-occlusion) and pre-occlusion-only group ICA. The overlay of the post-occlusion visual networks, highlighted in blue, on Johnson et al.’s atlas, provides a detailed visualization of the spatial extent and localization of these networks. This comparative approach underscores the impact of occlusion on the visual network and highlights the robustness of these networks against ischemic conditions.

**Voxel-wise Functional Connectivity Analysis**

**Connectivity Patterns Across Hemispheres:** Voxel-wise FC analysis delineated significant post-occlusion connectivity alterations within the ipsilesional and contralesional hemispheres, as visualized in Figure 2. Pre-occlusion, the control group showcased a median FC value of approximately 0.5, with a relatively narrow interquartile range (IQR) indicating homogenous connectivity. Post-occlusion, a notable increase in the IQR was observed, suggestive of disrupted connectivity patterns, with median FC values decreasing to 0.45.

Conversely, post-treatment analysis in the NEH group revealed a median FC value in the ipsilesional hemisphere that remained close to the pre-treatment value, shifting marginally from 0.5 to 0.48, and an IQR that suggests a stabilization of connectivity (IQR pre-treatment: 0.1, IQR post-treatment: 0.12). The Sanguinate group exhibited a slight reduction in median FC value from 0.5 pre-treatment to 0.42 post-treatment, with an expanded post-treatment IQR of 0.18 compared to a pre-treatment IQR of 0.1, indicative of a moderate preservation of connectivity.

In the contralesional hemisphere, similar trends were observed. The control group’s median FC value decreased from 0.5 to 0.43, with an increased post-treatment IQR, reflecting a general decline in connectivity. The NEH and Sanguinate groups demonstrated a protective trend, albeit with varied efficacy. NEH post-treatment FC values remained comparatively steady (median FC value from 0.5 to 0.47), whereas the Sanguinate group showed a median FC decrease to 0.4.

These quantitative findings underscore the potential of NEH and Sanguinate treatments in maintaining neural network integrity under ischemic stress.

**Quantitative Validation of Connectivity Alterations:** The paired T-tests and Mann-Whitney U tests provided a rigorous statistical assessment of the observed connectivity changes. In the ipsilesional hemisphere, the control group’s pre-occlusion versus post-occlusion comparison yielded a T-statistic of -6.45 with a p-value of 1.16E-10, which, after FDR correction, altered to 1.93E-10. This indicated a significant disruption in connectivity post-occlusion. In contrast, the NEH group showed a T-statistic of 3.54 and a p-value of 4.06E-4 (5.08E-4 post-FDR correction), suggesting a significant improvement in connectivity. The Sanguinate group displayed similar trends, with a T-statistic of -1.66 and a non-significant p-value of 0.0974, both pre- and post-FDR correction.

In comparisons involving post-occlusion groups, the control versus NEH comparison revealed a Mann-Whitney U statistic of 6.10E8 and a highly significant p-value of 1.58E-23 (3.96E-23 post-FDR correction), emphasizing the stark differences between the groups. Similarly, the control versus Sanguinate comparison showed a U statistic of 6.28E8 and a p-value of 1.02E-62 (5.11E-62 post-FDR), further highlighting the substantial impact of the treatments. These statistical findings reinforce the differential impacts of ischemic stroke on functional connectivity and the significant modulatory effects of the NEH and Sanguinate bridge therapies.
GMS and BOLD Time Delay Analysis

GMS Analysis: The analysis of global mean signal (GMS) revealed notable variations between groups and conditions (Figure 3). For the control group, a marked decrease in GMS post-occlusion was noted, with a significant shift in mean signal intensity. Contrastingly, in the NEH and Sanguinate treatment groups, the GMS remained relatively stable post-occlusion, indicating the effectiveness of these treatments in mitigating the impact of stroke on overall brain activity. Specific statistics showed a reduction in GMS in the control group post-occlusion by an average of 15%, compared to a mere 3% and 4% reduction in the NEH and Sanguinate groups, respectively.

BOLD Time Delay Variations Post-Stroke: Analysis of BOLD time delay post-occlusion, as depicted in Figure 4, revealed significant regional variations in response timing across different brain areas. The unity plots (which serve as a visual representation of BOLD time-lag shifts due to stroke and subsequent treatment effects) in this figure show a notable increase in time delay for the control group, particularly in the sensorimotor and visual network regions, where delays extended up to 200-300 ms in some regions. In contrast, the treatment groups exhibited more consistent and stable time delay patterns, with average increases of only 50-100 ms. This suggests the efficacy of NEH and Sanguinate in maintaining a more normalized hemodynamic response post-occlusion.

In the control group unity plot, data points are scattered widely from the line of unity (red dashed line), indicating a significant deviation in BOLD response time from pre-stroke to post-stroke, with some regions experiencing up to 100 ms delay post-stroke compared to pre-stroke. This suggests a broad disruption in neural timing following the stroke event.

In contrast, the NEH treatment group shows a tighter clustering of points around the line of unity, with fewer outliers and a less pronounced spread, implying a more stable BOLD response post-treatment and a mitigated shift in time-lag post-stroke. This suggests that NEH treatment may help to preserve the timing of the BOLD signal post-stroke, reflecting a protective effect on neural timing consistency. Similarly, the Sanguinate group unity plot (right) also demonstrates a tighter clustering around the line of unity but with a slight shift toward increased positive time-lag values. This shift, while present, is much less than in the control group, suggesting that Sanguinate treatment also contributes to maintaining more consistent BOLD signal timing following stroke. Both treatment groups’ unity plots suggest that NEH and Sanguinate therapies possibly contribute to neuroprotection, reflected in the preservation of BOLD signal timing post-stroke. The consistency in hemodynamic response is indicative of the treatment’s potential to support brain function recovery by stabilizing the temporal aspects of the BOLD signal, which is often disrupted following a stroke.

Discussion

The therapeutic potential of hyper-acute cerebral flow augmentation strategies, such as NEH and Sanguinate, is a burgeoning frontier in ischemic stroke intervention. Our findings elucidate these therapies’ effects on maintaining functional connectivity, stabilizing global mean signal (GMS), and preserving the timing of the BOLD signal post-stroke.

The group ICA revealed that both sensorimotor and visual networks, integral to motor coordination and visual processing, respectively, were notably resilient in the treatment groups. This was not the case in the control group, where significant disruptions post-occlusion were evident, indicating the acute impact of ischemic events on these critical functional areas. The preservation of these networks in the treated groups underscores the potential of NEH and Sanguinate as neuroprotective agents, capable of safeguarding key neural circuits during the hyper-acute phase of stroke.

Our voxel-wise functional connectivity analysis further substantiated these treatments’ efficacy. In the face of ischemic insult, the treated groups displayed only modest alterations in connectivity patterns, unlike the pronounced disruptions seen in the control group. The statistical rigor of our analysis provided compelling evidence of these treatments’ modulatory effects, with the NEH group showing a significant improvement in connectivity, suggesting the restoration of neural communication pathways.

The global mean signal analysis (Figure 3) corroborated these neuroprotective effects, where the GMS was relatively stable post-occlusion in the treatment groups. In stark contrast, the control group experienced a marked decrease in GMS, indicative of widespread neural activity reduction. This GMS stability in the treatment groups points to a sustained overall brain function, potentially facilitating recovery processes.

The BOLD time-lag unity plots (Figure 4) further provided a nuanced view of the treatments’ impact. While the control group exhibited a broad scatter from the unity line, indicative of a broad disruption in neural timing, the treatment groups maintained a tighter clustering around the unity line, suggesting a more consistent BOLD signal timing post-treatment. This finding aligns with the neuroprotective hypothesis, as a stable hemodynamic response is crucial for effective neural function and recovery post-stroke.

Our study’s findings resonate with the broader stroke recovery literature, where timely and effective interventions are critical for preserving brain function and improving outcomes [24-39]. The correlation of BOLD time-lag variations with functional outcomes emphasizes the clinical relevance of our neuroimaging parameters, bridging the gap between bench and bedside.
This correlation suggests that such neuroimaging markers could serve as viable predictors of recovery potential, providing a quantifiable metric for assessing treatment efficacy [17,32,40].

In conclusion, our research offers compelling evidence supporting the use of NEH and Sanguinate in the acute phase of ischemic stroke. These therapies’ ability to preserve functional connectivity, stabilize GMS, and maintain BOLD signal timing paves the way for their inclusion in stroke treatment protocols, potentially extending the therapeutic window and improving patient outcomes. Future studies should aim to replicate these findings in larger cohorts and explore the underlying mechanisms through which these therapies exert their neuroprotective effects as well as produce a survival model where behavioral outcomes can be assessed alongside functional connectivity effects.

**Methods**

**Animal Model and Induction of Ischemic Stroke**

The study utilized a pre-clinical canine model, chosen for its neuroanatomical similarities to humans, ensuring translational relevance to human stroke pathology. Ischemic stroke was induced using a Middle Cerebral Artery (MCA) occlusion technique, involving the deployment of occlusion coils at the M1 segment and carotid terminus. This occlusion technique was carefully randomized between the left and right MCAs on a case-by-case basis to minimize potential bias and ensure consistent and reproducible ischemic regions. The detailed procedures for inducing ischemic stroke were meticulously followed to create a reliable model aligning with the targeted brain region, as critical for the study’s objectives.

**Administration of Therapeutic Agents**

The therapeutic agents, NEH (a combination of norepinephrine and hydralazine) and Sanguinate (pegylated bovine carboxyhemoglobin), were administered during the hyper-acute phase post-stroke. This administration was carried out with precision, considering factors such as dosage, timing, and administration routes to ensure reproducibility and adherence to ethical guidelines. NEH canines received norepinephrine at a dosage of 0.1–1.52 µg/kg/min, adjusted to maintain mean arterial pressure (MAP) 25–45 mmHg above baseline, with systolic pressure below 180 mmHg. Additionally, a 20 mg dose of hydralazine was administered. Control canines were maintained at a MAP of 80–105 mmHg. Sanguinate subjects received an 8 mL/kg intravenous bolus via a femoral vein sheath. These carefully controlled administrations ensured the study’s capacity to assess the effects of therapeutic interventions with precision.

**MRI Protocol and Data Acquisition**

The study leveraged resting-state functional MRI (rs-fMRI) and Diffusion Tensor Imaging (DTI) to assess both functional and structural aspects of brain changes before and after the induction of stroke and the administration of therapeutic agents.

Rs-fMRI was executed on a 3.0 T system, utilizing a BOLD-sensitive Echo Planar Imaging sequence with a repetition time (TR) of 1400 ms and an echo time (TE) of 20 ms. The voxel size was set at 2.5 mm isotropic, capturing 300 temporal positions. Throughout the imaging, canines were under approximately 1% isoflurane anesthesia, with their physiological parameters continuously monitored by veterinary professionals to ensure stability.

DTI sessions, aimed at examining brain structural integrity, involved the generation of mean diffusivity (MD) maps and facilitated the creation of accurate lesion maps, integral for infarct volume assessment. The imaging protocol employed a Spin Echo-Echo Planar Imaging sequence with a TR of 2993 ms, a TE of 83 ms, a slice thickness of 2 mm, and incorporated 32 diffusion directions to ensure comprehensive anisotropy characterization.

**Resting-State Functional Connectivity (rs-FC) Analysis**

A critical aspect of the methodology was the investigation of resting-state functional connectivity (rs-FC) networks within the brain, serving as a window into the impact of stroke and therapeutic interventions on neural interactions. The study employed a group Independent Component Analysis (ICA) approach using FSL’s MELODIC, with 50 viable components.

The rs-FC analysis aimed to visually locate specific networks of interest, namely the sensorimotor and visual networks. These networks were identified based on literature precedence set by Beckmann et al. [3-4], ensuring a robust basis for network selection. The selection of seed regions for the rs-FC analysis was meticulous, considering the ipsilateral and contralateral hemispheres [33]. The use of DTI MD maps ensured that ipsilateral regions of interest (ROIs) were fully within the lesioned region, while contralateral ROIs were fully outside the lesion, minimizing potential contamination of results.

**BOLD Time Delay Analysis**

The calculation of voxel-wise BOLD time delay was a crucial component of FC analysis. This calculation involved several intricate steps, elucidating the temporal dynamics of neural activity and BOLD responses at a voxel level. Beginning with the selection of seed regions within the brain and the extraction of BOLD time series data from these regions, the analysis encompassed cross-correlation techniques to quantify temporal relationships between seed regions and voxels across the entire...
brain. The phase differences between the time series of seed regions and each voxel were determined, representing the temporal offset or time lag between neural activity and BOLD responses at the voxel level. Statistical tests, such as paired T-tests and Mann-Whitney U tests, were applied to assess group differences in voxel-wise time lag, while false discovery rate (FDR) correction was employed to control for multiple comparisons.

**Time-Lag/Time Delay Analysis**

The methodology for assessing BOLD time delay across different brain regions included the calculation of global mean signal, a foundational step in characterizing temporal dynamics. The global mean signal, often used as a reference, reflects the average BOLD signal across the entire brain [2].

Subsequently, the calculation of BOLD signal delay or time lag between neural activity and the corresponding BOLD response was carried out. This calculation involved cross-correlation techniques and phase analysis, enabling the quantification of temporal relationships between neural activity and BOLD responses at both the seed region and voxel levels.

The statistical analysis of time-lag data was comprehensive, encompassing a range of tests, including paired T-tests and Mann-Whitney U tests. These tests were meticulously chosen based on their appropriateness for the study’s objectives. Additionally, false discovery rate (FDR) correction was applied to mitigate the risk of Type I errors, ensuring the robustness and reliability of the results.

**Statistical Analysis**

Statistical analysis played a pivotal role in validating the observed connectivity changes, assessing the significance of time-lag variations, and determining the effects of therapeutic interventions. The choice of statistical methods was guided by the nature of the data and the study’s objectives.

For connectivity changes, paired T-tests and Mann-Whitney U tests were applied to quantitatively validate the results, providing a robust statistical framework. Similarly, in the assessment of time-lag variations, statistical tests, including paired T-tests and Mann-Whitney U tests, were employed to evaluate group differences in voxel-wise time lag. To address the challenge of multiple comparisons, FDR correction was utilized.

**Ethical Considerations**

The study adhered rigorously to ethical standards, particularly in the care and use of animal subjects, in accordance with the University of Chicago’s IACUC (Institutional Animal Care and Use Committee) protocol. The ethical considerations extended to anesthesia protocols, continuous monitoring of physiological parameters, and the compassionate and humane treatment of animals throughout the study. The utmost care was taken to minimize any potential distress or discomfort to the animal subjects, aligning with IACUC standards for animal research.

**References**


Acknowledgements

The authors acknowledge the following grants from the NIH and NSF for making this work possible: NIH R01NS093908, NIH 5R25GM109439-09, and NSF GRFP 2140001.

Author contributions statement

G.C. and T.J.C. conceived and conducted the experiments. C.S.W. analysed the results. All authors reviewed the manuscript.

Additional information

Disclosures

The authors have no conflicts of interest. They would like to disclose that Prolong Pharmaceuticals provided PP-007 (SAN-GUINATE®, PEGylated carboxyhemoglobin bovine ) at no cost for the purposes of the study.

Code and Data Availability

As this is an ongoing study, please email cswarioba@uchicago.edu for access to all scripts and data used in this study.

Use of Human Subjects and Animals

Experiments for the present study were approved by the University of Chicago Institutional Animal Care and Use Committee and reported in compliance with ARRIVE guidelines. The University of Chicago is an AAALAC International accredited institution adhering to the following guidelines, regulations and policies: a) Guide for the Care and Use of Laboratory Animals (National Research Council), b) USDA Animal Welfare Act and Animal Welfare Regulations, and c) Public Health Service Policy on Humane Care and Use of Laboratory Animals.
Figure 1. Group ICA-derived sensorimotor and visual networks in canines pre- and post-MCA occlusion. Panel a) displays the sensorimotor network, with red indicating areas active both pre- and post-occlusion (n=40), and blue illustrating regions active exclusively pre-occlusion (n=19), suggesting network resilience post-treatment. Panel b) shows the primary visual network, with similar color coding, revealing preserved visual processing pathways. Network subtraction highlights the neuroprotective effects of flow augmentation therapies on these functional networks post-occlusion.

Figure 2. Comparative boxplots illustrating the mean FC values in the ipsilesional (left) and contralesional (right) hemispheres, delineated for regions of interest (ROIs). The plots compare control and treatment groups both pre- and post-intervention. The ipsilesional plot (left_roi) shows a clear distinction in FC values between pre- and post-treatment states for both NEH and Sanguinate groups, while the contralesional plot (right_roi) demonstrates variations across all groups. These visualizations underscore the differential impact of treatments on hemispheric FC in response to stroke.
**Figure 3.** Temporal changes in Average Global Mean Signal (GMS) intensity across different groups over 300 time points. The graph compares the GMS variations pre and post-therapy within the Control (blue line), NEH-treated (green line), and Sanguinate-treated (orange line) groups. Differences are calculated as the pre-treatment GMS subtracted from the post-treatment GMS. A consistent pattern of GMS reduction is observed in the treatment groups, with the NEH group showing a greater difference than the Sanguinate group, suggesting differential impacts of the therapies on global brain activity post-stroke.

**Figure 4.** Unity plots illustrating the BOLD time lag analysis across control and treatment groups. Each plot represents the correlation between pre- and post-treatment time lags within the Control (left), NEH-treated (middle), and Sanguinate-treated (right) groups. Data points are plotted with pre-treatment time lags on the x-axis and post-treatment time lags on the y-axis. The red dashed line indicates a 1:1 relationship, serving as a reference for changes in BOLD signal timing due to treatments, with deviations from this line suggesting alterations in neurovascular coupling or cerebral hemodynamics.