SUPPLEMENTAL MATERIAL

Deferoxamine, Cerebrovascular Hemodynamics and Vascular Aging: Potential Role for HIF-1 Regulated Pathways

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Supplemental Methods

Subjects and Experimental Design

Forty eight healthy subjects (45 ± 19 years, range 19 to 80 years old) volunteered to participate in the study. Young participants were 18-45 years-old and old participants were 55-80 years old. All volunteers were carefully screened with a medical history, physical examination, and electrocardiogram to exclude any acute or chronic medical conditions. All volunteers were free of any diagnosed disorders including neurodegenerative disorders, asthma, diabetes mellitus, intracranial or carotid stenosis, hepatic disease, renal disease, bone marrow suppression, cardiac disease, hypertension, heart failure, iron deficiency, any history of malignancy, history of head trauma, subarachnoid hemorrhage, central nervous system vasculitis, multiple sclerosis, migraines, seizures, sickle cell disease or trait, or cardiac arrhythmias. They were also excluded if they had any cognitive impairment. Participants were not any vasoactive medications. We also excluded pregnant women. Subjects were nonsmokers and refrained from alcohol for at least 12 hours. The study was approved by the institutional review board, and all subjects gave informed consent.

The study was designed as a blinded, randomized cross-over study. Each subject was admitted twice to the Clinical Research Center (CRC) at the Brigham and Women's Hospital. Each admission was for 24 hours and scheduled at least 30 days apart (see Supplemental Figure). Subjects were randomized to receive either DFO (dissolved in 5% Dextrose-normal saline) or placebo (PLB: 5% Dextrose-normal saline) during their first admission. Randomization was done by the CRC pharmacy, and DFO or placebo solutions were delivered in unmarked containers. Thus, all investigators were blinded to the infusion. During their second admission, volunteers crossed-over to the other infusion arm. That is, those who received DFO on their first admission received placebo, and vice versa. Thirty days separation between the two admissions ensured sufficient wash-out between the two admissions.

Participants were admitted between 5-7 pm the night before the study day. Study protocols are depicted in Supplemental Figure. On admission, an intravenous catheter was placed in each arm; one for infusion and the other for blood draw to assay for VEGF and EPO levels. Normal saline was infused at a rate of 20cc/hr through each line and continued until the infusion (DFO/PLB) started at 9 am the next day through the "infusion line" at 70cc/hr. The "blood draw line" was continuously infused with normal saline at 20cc/hr.

The next morning (study day), first meal was served at 6 am. Subject instrumentation (below) was started at 7 am. After baseline data collection (t_0) was completed the infusion (DFO/PLB) was started at 9 am. Second meal was served at 10 am and first time-point (3^{rd}hr, t_3) data collection was completed at 12 noon. Third meal was served at 1pm and second time-point (6th hour; t_6) data collection was completed at 3 pm and infusion (DFO/PLB) ended. Fourth meal was served at 4 pm. Normal saline infusion at 70cc/hr was continued in the "infusion line" until 6 pm when the study ended and post-infusion data collection (9th hour; t_9) was completed at 6 pm. The dose of DFO infused was 60mg/kg over 6 hours. Subjects were discharged home after a medical evaluation (see the supplemental Figure). All meals and fluid intake (including the first night meal and fluids) where exactly matched throughout each study day and during the two admissions. Between studies subjects remained instrumented, but were allowed to walk around the unit as needed, watch TV or read a book. All measurements were performed while subjects were in seated position.

Because caffeine intake can have a marked impact on cerebrovascular function, we banned caffeine containing food/beverages throughout both study admissions.

Measurements and Assessment of Cerebrovascular Function

Photoplethysmographic mean arterial blood pressure (MAP) was recorded continuously on the finger (Portapres, Finapres Medical Systems, Netherlands), and cerebral blood flow velocity was measured using the transcranial Doppler ultrasound (MultiDop X, DWL-Transcranial Doppler System Inc., Sterling, VA) bilaterally in the M1 segment of the middle cerebral arteries (MCA) at a depth of 50 – 65 mm. The diameter of the MCA remains relatively constant with changes in blood pressure and arterial gases within physiologic range.[1] Thus, mean flow velocity (MFV) was used as a surrogate for flow. Expired CO_2 was continuously monitored by an infrared CO_2 analyzer (VacuMed #17515 CO_2 Analyzer Gold Edition, VacuMed Medical, Ventura, CA) connected to a nasal cannula. All signals were digitized and stored at 500 Hz (DI-720 Series Data Aquisition Systems and Windaq Software, DATAQ Instruments, Inc., Akron, OH).

To assess cerebrovascular function, we quantified its three main components: vasoreactivity, autoregulation, and neurovascular coupling. Cerebrovascular reactivity (VR) was assessed based on the slope of the linear relation between end-tidal CO_2 and MCA flow velocity. This provided a measure of change in MFV per unit change in end-tidal CO_2. Participants were asked to breathe room air for 2 minutes, then inspire a gas mixture of 8% CO_2, 21% O_2, and 71% nitrogen for 2 minutes, and then mildly hyperventilate in room air to an end-tidal CO_2 of approximately 25 mmHg for 2 minutes. Room air consists of 21% O_2 and 0.003% CO_2 with balance in N_2. Cerebral vasoreactivity was then assessed as the linear slope between the change in $EtCO_2$ and cerebral blood flow. None of the volunteers experienced any hypoxia. $EtCO_2$ was measured using a gas sampling interface designed specifically for monitoring CO_2 and O_2 in non-intubated patients. The interface consists of a small tube with a nasal adapter that is inserted into one nostril. All subjects were instructed to breathe in and out only through their nose for the entire duration of the protocols to measure, $EtCO_2$ was continuously sampled through this narrow tube, and analyzed via the VacuMed infrared analyzer. During CO_2 re-breathing, CO_2 was administered through a non-re-breather mask held over the subjects' mouth and nose while the subject continued to breathe in and out through the nose. CO_2 was administered always at the same rate on the gauge and using the same gas mixture. Cerebral autoregulation was assessed based on transfer function analysis of the relationship between pressure and flow in the MCA as described before.[2;3] The 50-Hz waveforms of arterial pressure and cerebral blood flow were recorded for at least 5 minutes, and decimated to 5 Hz and low pass filtered with a cutoff of 0.4 Hz. Filtering, as opposed to interpolated means, was used to provide signals that were independent of possible changes in the electromechanical delay from R-wave to generation of a pressure–flow pulse. Power spectral density estimates were calculated by Welch average modified periodogram method.[4] To that end, the filtered time series was divided into 5 segments of equal length that overlapped by 50%. The signals in each segment were linearly detrended, smoothed through a Hamming window, and fast-Fourier transformed. Subsequently, spectral power estimates were averaged across all windows, and transfer function parameters were derived from the cross spectrum of the pressure signal with the complex conjugate of the cerebral flow velocity signal. Confidence intervals and precision of estimates for the transfer function were derived based on the level of coherence.[5] We examined coherence, gain, and phase relation between arterial pressure and cerebral flow velocity across 0.03 – 0.07 Hz range, within which autoregulation has been shown to be most active.[2;6] Coherence is analogous to linear regression coefficient, and indicates how linearly the fluctuations in pressure are transmitted to cerebral circulation (higher coherence indicates less

effective autoregulation). Transfer function gain provides a measure of how much the cerebral blood flow changes per change in pressure (higher gain indicates less effective autoregulation), and transfer function phase provides a measure of the temporal course between arterial pressure and cerebral blood flow responses (lower phase indicates shorter responses, and thus, less efficient autoregulation). Finally, neurovascular coupling was assessed at baseline (t_0) and at t_6, as the percent change in MFV during a 2-Back task as described before.[2;7] The mean percent change in MFV for each MCA was calculated as the percent difference between the MFV during the 2-back and its corresponding 0-Back control period. Neurovascular coupling tasks were done on a computer with the subject clicking a button marked "yes" or "no" for each target letter. There were three tasks: 0-back (which served as a control task) instructed subjects to click "yes" every time they saw the letter "X" presented on the screen, and click "no" for any other letter. The 1-back task required subjects to click "yes" every time they saw the same letter repeated one letter later, for example A – A, and click "no" for other letters. The 2-back task required subjects to click "yes" every time they saw the same letter repeated two letters later, for example A – B – A, and click "no" for other letters. The computer recorded each response in order to track subject cooperation and performance. The 0-back control task was alternated between each 1-back and 2-back trial every 60 seconds for a total of 10 minutes

Biomarkers of HIF-1 activation

Ten milliliters of blood was collected from each subject in a sterile glass tube containing 100 IU of preservative-free heparin (Sigma, St Louis, MO, USA) at each time point. Plasma EPO and VEGF were quantified by Meso Scale Discovery Custom Multiplex and Sector Imager 2400 (MSD, Gaithersburg, MD). The calibrators' range was 10 IU/ml – 0.038 mIU/ml for EPO and 200 ng/ml – 0.763 pg/ml for VEGF. The lower limit of detection varied from 0.514 to 5.35 mIU/ml for EPO and 7.38 to 22.5 pg/ml for VEGF, with an average of 1.47 and 12.18, respectively. To block non-specific binding, the plates were pre-incubated with 150 µl/well blocking solution (casein-supplemented TRIS buffer provided by MSD) for one hour followed by washing with 0.05% Tween-20 supplemented PBS. A diluent containing BSA, serum and casein provided by MSD was then added at 25 µl/well and equal volumes of calibrators and samples were added on top for two hours followed by another wash step, incubation with sulfo-tag labeled detection antibodies for two hours, a wash, and a reading within 5 minutes from adding a read buffer (all reagents provided by MSD). To assess inter-assay coefficient of variation, a serum pool was generated as a quality control, and an aliquot of it repeatedly run on each plate along with the test samples. The coefficient of variation was calculated as the [standard deviation/average]*100 of two quality control relative luminescent readings per plate and was 26% for EPO and 10% for VEGF. Raw readings were converted to concentrations using the MSD Workbench software.

Statistics

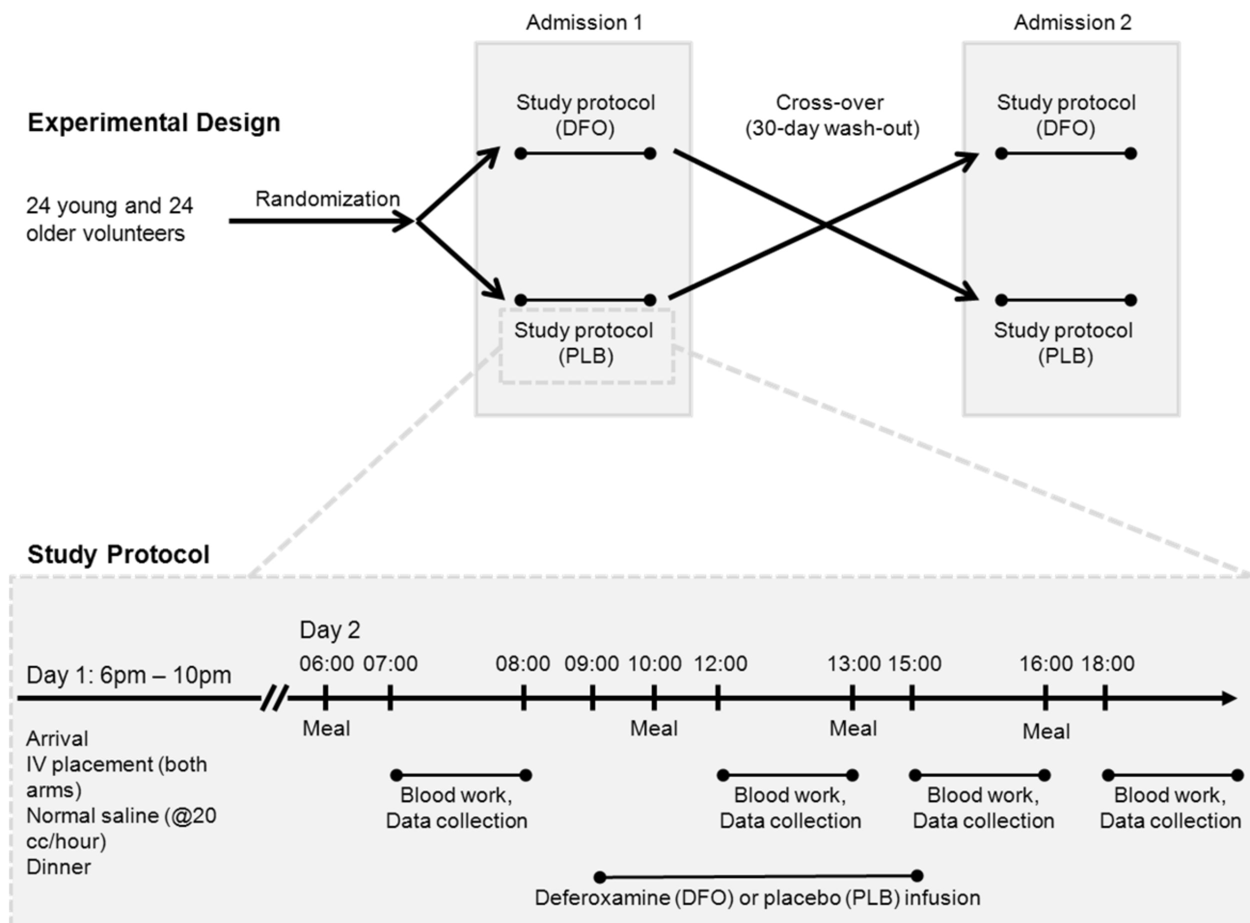
Log transformations were applied to spectral powers and the inverse hyperbolic tangent to coherence to provide estimates with asymptotically standard distributions.[8] The Box-Cox transformation was applied to all other data to ensure normality when necessary. However, for ease of interpretation, all average values and standard errors are presented as standard units.

For statistical analyses, transfer function gain and phase were weighted by their precisions to obtain the most accurate means for statistical analysis. Stationary random process theory yields an estimate for the precision of transfer function gain and phase based on coherence.[5] Therefore, gain and phase were weighted by their precision (which is determined by the level of coherence, data length, and transfer function parameters, including window size, length, and

overlap) to obtain the most accurate estimate of means for statistical analysis. In this way, unreliable estimates received appropriately small weights when group averages and statistics were computed.[9]

There were no significant differences in the right and left MCA MFV or measures of cerebrovascular function (vasoreactivity, coherence, and transfer function gain and phase, p > 0.20 for all comparisons). Therefore, to maximize reliability of our statistical analysis and to minimize the risk of committing Type-I error, we treated measures derived from right and left MCA as repeated measures for each individual. Placebo infusions were done in order to control for the potential effect of increased plasma volume on blood pressure, flow, and vascular function measures. All variables of interest were tested for significance via a linear mixed-effect model with age (young vs old), treatment (PLB vs DFO) and time (baseline, 3- and 6-hours of infusion, and 3-hours post-infusion) (including their interaction) as fixed effects and subject and the side of measurement (nested in subject) as random effects. All effects were considered statistically significant at p<0.05 level. All descriptive statistics were reported as mean + standard error.

Supplemental Figure Schematic description of the experimental design and study protocols



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