A Novel Neuroprotective Strategy Using Methylene Blue---A Longitudinal MRI Study

Qiang Shen[1], Fang Du[1], Shiliang Huang[1], Yash Vardhan Tiwari[1], and Timothy Q Duong[1]

[1]*Research Imaging Institute, University of Texas Health Science Center at San Antonio, San Antonio, Texas, United States*

INTRODUCTION Methylene blue (MB), routinely used to treat malaria, methemoglobinemia, and cyanide poisoning[1], has recently been shown to reduce neurobehavioral impairment in animal models of Parkinson's Disease[2] and cognitive decline in Alzheimer's.[3] MB has unique energy-enhancing and antioxidant properties.[1-3] MB's auto-oxidizing property acts as an electron cycler that allows MB to redirect electrons to the mitochondrial electron transport chain, thereby enhancing ATP production and promoting cell survival. In vitro studies have firmly established that MB enhances ATP production and oxygen consumption.[4,5] In bypassing complex I-III, MB also reduces reactive oxygen species production from the mitochondrial electron transport chain, which has antioxidant effect. MB thus has the potential to minimize ischemic injury, reperfusion injury, and maximize functional recovery. It has been recently demonstrated that MB: i) has protective effects against glutamate cytotoxicity and oxygen glucose deprivation (OGD)/reoxygenation in vitro, and ii) substantially reduces infarct size in transient (60-min) focal cerebral ischemia in rats by histology.[6] As the next logical step, in this work, we evaluated MB's neuroprotective effects using MRI to longitudinally evaluate ischemic evolution. Comparisons were made with functional changes using neurological assessments.

METHODS Twelve male Sprague Dawley rats (250-300g) were subjected to 60-min transient MCA occlusion using intraluminal suture occlusion method.[7] Using a randomized and double-blinded experimental design, either vehicle or MB was administered (1 mg/kg, i.v. infusion over 30 mins) immediately after reperfusion, and again 3-hr post-occlusion (0.5mg/kg), day-2 and day-7 (1 mg/kg).

MRI experiments were performed on a 7-T/30-cm magnet. Quantitative CBF (cerebral blood flow) and ADC (apparent diffusion coefficient) were measured using continuous arterial spin labeling and diffusion-weighted EPI.[7] T2 maps were acquired using fast spin echo sequence[7] in day-2. Behavioral tests were performed at acute phase, day-1, 2, 7 and 28. Animals were scored neurologically according to a 6-point scale (0 = no deficit, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = falling to the left, 4 = no spontaneous walking with a depressed level of consciousness, and 5 = dead).

Based on 30-min ADC and CBF maps, ischemic core, perfusion/diffusion mismatch and normal tissues were classified using automated clustering ISODATA method.[8] Initial lesion volumes were defined by the core tissue volumes. Final infarct volumes were derived from day-2 T2 maps using threshold of mean T2 value of normal hemisphere plus two times of standard deviation. Edema correction was applied.[9] Day-2 data was co-registered to acute phase data[10] for pixel-by-pixel tissue fate tracking. Paired or equal variance t-test was used for comparison between initial lesion and final infarct or between treated and control groups. A P-value of 0.05 was taken to be statistically significant. Data showed in figures and texts are mean ± SEM.

RESULTS Initial ADC-defined lesion volume at 30 mins and final T2-defined infarct volume at 2 days after stroke were evaluated (**Figure 1**). The initial lesion volumes of the vehicle- and MB-treated group were *not significantly different* (P=0.92). In the vehicle-treated group, the initial lesion volume grew larger at day 2 (19±6%, N=6, P=0.03). By contrast, in the MB-treated group, the initial lesion volume became smaller at day 2 (-17±5%, N=6, P=0.02). The MB-treated infarct volume was significantly smaller than vehicle-treated infarct volume at day 2 by 30% (P=0.03).

We further showed that MB salvaged more initial core pixels compared to controls (22±3% *vs* 11±3%, P=0.03), and MB also salvaged more mismatch pixels compared to vehicle (83±3% *vs* 61±8%, P=0.02) (**Figure 2**). The mean NINDS stroke scores of the MB- and vehicle-treated rats were, respectively, 1.5 and 2 at day 2 (P=0.04), and 1.0 and 1.7 at day 7 (P=0.01), indicative of improved neurological status.

CONCLUSIONS Our results showed that MB significantly reduces infarct size and behavioral deficit in the 60-min cerebral ischemia in rats. Such neuroprotective effect is consistent with MB's unique properties as a metabolic enhancer and an antioxidant. Future studies will investigate different occlusion durations, chronic stroke, embolic stroke model with combination therapy with rtPA, as well as incorporate fMRI and other MRI measurements. MB is already clinically approved for other indications, which should enable speedy translation to clinical trials.

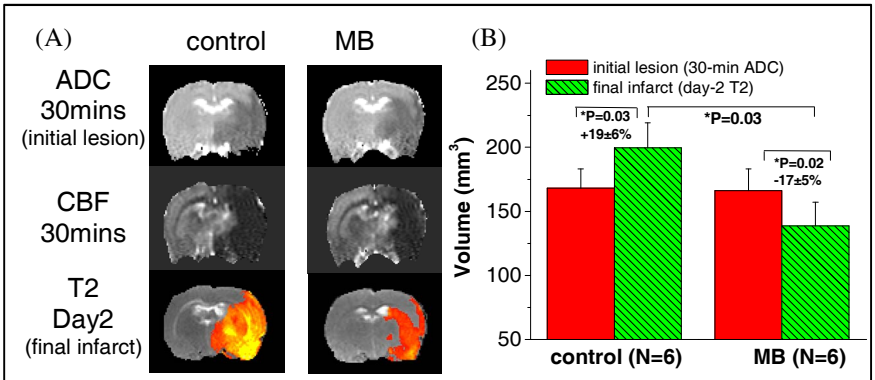

Figure 1. (A) ADC, CBF maps at 30 mins post-occlusion and T2 maps on day-2 of vehicle- and MB-treated rats subjected to 60-mins MCAO. **(B)** Initial lesion (30 mins) and final infarct (day 2) volume of vehicle- and MB-treated rats subjected to 60-mins MCAO (n = 6, ±SEM).

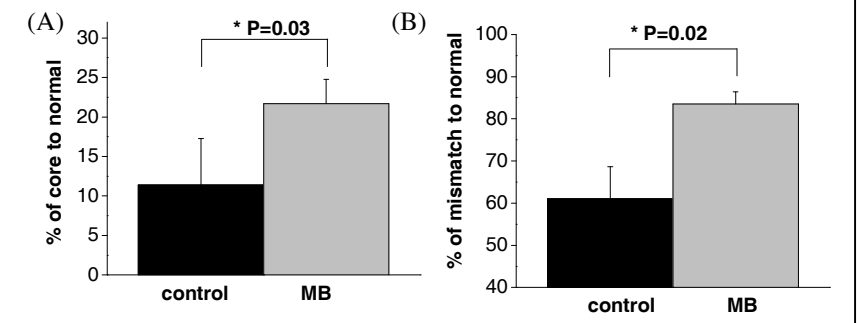

Figure 2. Percentage of core (A) and mismatch (B) tissues salvaged with and without MB

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