The Effects of Methylene Blue on Autophagy and Apoptosis in MRI-defined Normal Tissue, Ischemic Penumbra and Ischemic Core

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Target Audience Researchers in stroke and neuroprotection

INTRODUCTION We recently found that methylene blue (MB) decreases infarct size and behavioral deficit following experimental stroke [1]. In this study, we probed the underlying molecular mechanisms of neuroprotection of MB using western blot analysis of apoptotic and autophagic cascades in different tissue. Multiple MRI was used to define different types of at risk and ischemic tissue for western blot analysis.

METHODS: Male SD rats (250-350g) underwent 60min middle cerebral artery occlusion (MCAO) under 2.0% isoflurane, sham surgery (n=5), control (n=10) or MB (n=10) was administered at 30mins, 6hrs and 15hrs (1mg/kg each). CBF and ADC MRI were acquired at 30 and 90mins and T_2 maps were acquired at 24hrs on a 7T [1]. Initial lesion volumes were defined at 30mins and final infarct volume was defined at 24 hrs as described in [1]. Edema correction was applied.

Based on the 30min ADC and CBF maps plus the 24hrs T_2 map, three ROIs were identified as (A) the perfusion-diffusion mismatch area salvaged by reperfusion, (B) the perfusion-diffusion mismatch area not salvaged by reperfusion, and (C) the ischemic core. At 24hrs, tissues from these three regions were processed using a standard western blot protocol [2] with antibodies against LC3, caspase-3, p53 and Beclin-1. Comparisons of western blot analysis were made with behavioral scores (neurological scores and foot fault analysis). T-test was used for statistical comparison with a p value < 0.05 taken to be statistically significant.

RESULTS AND DISCUSSION: **Figure 1** shows typical CBF and ADC images from a control and a MB animal at 30mins and 90mins post-MCAO. The initial ADC volumes at 30mins *before* treatment were not statistically different between the control and MB groups as expected (159±13 vs 150±24 mm^3, p>0.05). However, the final T_2 lesion volume at 24hrs was smaller in the MB compared to control group (58±8 vs 106±14 mm^3, p<0.05). These results indicated that MB decreased infarct volume at 24hrs, consistent with a previous study [1]. After reperfusion at 90mins, CBF values in both ROI-B and C (normalized CBF: 1.11±0.19 and 1.00±0.12, respectively) of the MB group were higher than those of the vehicle group (0.59±0.08 and 0.70±0.11, p<0.05 respectively), indicating that MB increased CBF after reperfusion. At 24hrs, the same ROIs in the MB group showed less hyperperfusion (which is associated with worse outcome [3]) than that of the vehicle group.

Analysis of western blot result indicated that MB increased autophagy and inhibited apoptotic cell death in ROI-A and B, but not in ROI-C (**Figure 2A**). Our data are the first to demonstrate that MB inhibits the apoptotic p53-Bax-Bcl2-caspase3 pathway, suppressing apoptotic cell death in the penumbral ROIs, thereby preventing the transition into infarct and decreasing overall infarct volume (**Figure 2B**). MB also modulates autophagic cell death program, which is mediated by inhibition of p53, which subsequently activates both AMPK and TSC2 causing inhibition of mTOR in the penumbral regions (**Figure 3**).

Behavior test results also showed improved sensorimotor function recovery in the MB compared to vehicle group (data not show).

CONCLUSIONS: This study demonstrated that MB-induced neuroprotection in ischemic stroke is mediated by enhancing autophagy and suppressing apoptosis. Multimodal MRI enabled the extraction of different tissue types for western blot analysis. The combined use of western blot analysis and multimodal MRI shred lights on the underlying molecular mechanisms of MB neuroprotection in different ischemic tissue types.

REFERENCES: [1] Shen al, PLosOne 2013. [2] Congdon EE et al, Autophagy 2012. [3] Shen et al. JCBFM 2011.

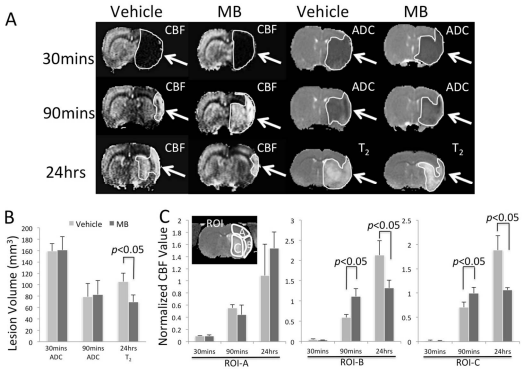

Figure 1. MB reduces infarct volume and CBF deficit. **(A)** Typical ADC, CBF and T2 maps, **(B)** lesion volumes, and **(C)** CBF from 3 ROIS (insets) at multiple time points post-occlusion of the vehicle and MB groups.

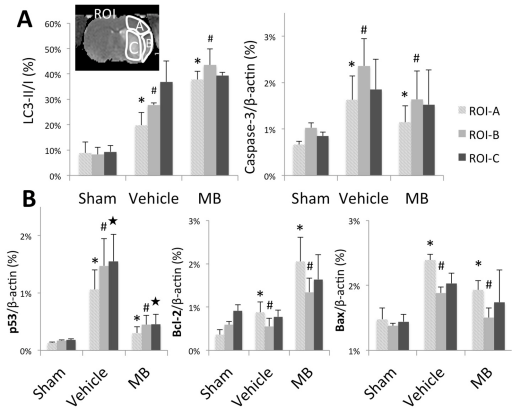

Figure 2 (A) Western blot analysis of autophagic marker LC3-II/I ratio, and apoptotic marker Caspase-3 in sham, vehicle and MB groups. (B) Western blots of p53, Bcl-2 and Bax 24hrs after stroke in the same ROI of the sham, vehicle and MB group. *p<0.05 versus vehicle in ROI-A. #p<0.05 versus vehicle in ROI-B. ★p<0.05 versus vehicle in ROI-C. Values are mean±SEM, n=5 in each group.

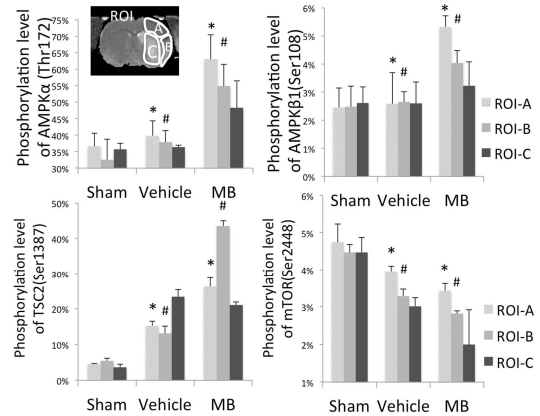

Figure 3. Western blots of AMPK through the hyper-phosphorylated of AMPKα at Thr172 site and AMPKβ1 at Ser108 site 24hrs after stroke of vehicle and MB group. Western blots of phosphorylation level of TSC2 at site Ser1387 and mTOR at site Ser2448 24hrs after stroke of vehicle and MB group. *p<0.05 versus vehicle in ROI-A. #p<0.05 versus vehicle in ROI-B.