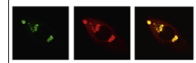


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Research Report

Methylene blue treatment delays progression of perfusion–diffusion mismatch to infarct in permanent ischemic stroke



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ABSTRACT

Stroke is a leading cause of morbidity and mortality in the world. Low-dose methylene blue (MB), which has been used safely to treat methemoglobinemia and cyanide poisoning in humans, has energy enhancing and antioxidant properties. We tested the hypothesis that methylene blue treatment delays progression of at-risk tissue (ca. perfusion–diffusion mismatch) to infarct in permanent middle cerebral artery occlusion in rats at two MB treatment doses. Serial MRI was used to evaluate MB treatment efficacy. The major findings were: (i) MB significantly prolonged the perfusion–diffusion mismatch, (ii) MB mildly increased the CBF in the hypoperfused tissue, (iii) MB did not change the final infarct volume in permanent ischemic stroke, and (iv) there were no dose-dependent effects on mismatch progression for the 1 and 3 mg/kg doses studied. This neuroprotective effect is likely the result of sustained ATP production and increased CBF to tissue at risk. This work has the potential to readily lead to clinical stroke trials given MB's excellent safety profile.

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1. Introduction

Stroke is the second leading cause of death and the leading cause of disability in the world (World Health Organization, 2011). Recombinant tissue plasminogen activator (rt-PA) remains the only approved ischemic stroke therapy available to the masses. Moreover, rt-PA treatment only reaches less than 5% of patients due to its narrow therapeutic window and its risk of intraparenchymal hemorrhage. There is an urgent

need for new neuroprotective therapies that can extend the thrombolytic therapeutic window.

Methylene blue (MB) is a grandfathered FDA drug that was first synthesized at the end of the 19th century and has been used to treat methemoglobinemia and cyanide poisoning. Low-dose MB has an excellent safety profile. MB has redox recycling properties in that it acts as an electron cyclor and facilitates electron transfer in the mitochondrial electron transport chain from NADH to cytochrome c with resultant

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effect of ATP production. By rerouting electrons directly to cytochrome c, and bypassing complexes I–III, MB also minimizes oxygen free radical production in the mitochondrial electron transport chain, especially under oxidative stress conditions. The energy-enhancing and antioxidant properties of MB could have potential neuroprotective effects.

Recently, low dose MB has been shown to exhibit therapeutic effects in a number of neurological disorders. MB reduces neurobehavioral impairment in optic neuropathy (Rojas et al., 2009; Zhang et al., 2006), Parkinson's disease (Rojas et al., 2012) and Alzheimer's disease (Congdon et al., 2012; O'Leary et al., 2010) in animal models. Clinical studies have shown that MB can slow the progression of Alzheimer's disease (Wischnik and Staff, 2009; Wischnik et al., 2008). MB was also shown to have neuroprotective effect in transient ischemic stroke models (Shen et al., 2013; Wen et al., 2011) and in a traumatic brain injury model in rats (Watts et al., 2014).

Magnetic resonance imaging offers a non-invasive means to track the progression of ischemic brain injury in a longitudinal fashion. T2-weighted MRI is widely used to visualize edema and define final infarct volume (Shen et al., 2005). Perfusion-weighted MRI can measure cerebral blood flow (CBF) at the tissue level, allowing for the detection of tissue with reduced perfusion that is at risk of ischemic brain injury. Diffusion-weighted MRI (DWI), which measures water motion, is very sensitive to early ischemic brain injury in contrast to computed tomography and T2 MRI (Moseley et al., 1990). As such DWI has become the method of choice for early detection of ischemic brain injury. Although the underlying biophysical mechanisms of DWI signal contrast is not fully understood (Duong et al., 1998), the combined use of perfusion and diffusion MRI are now widely used to distinguish reversible from irreversibly ischemic brain injury, and to guide acute stroke treatment in preclinical and clinical settings (Astrup et al., 1981; Schlaug et al., 1999).

The goals of the current study were: (i) to test the hypothesis that methylene blue treatment delays progression of perfusion–diffusion mismatch to infarct in permanent ischemic stroke in rats, and (ii) to evaluate the effects of two treatment doses. A randomized, double-blind and placebo controlled design was used to avoid bias. MRI was used to verify the presence of

mismatch at the hyperacute phase, to exclude incomplete occlusion, and ensure similar initial lesion sizes between the two groups before treatment. Such subject selection, which has been demonstrated to be critical in clinical stroke treatment trials, would not have been possible with terminal histological measurements.

2. Results

2.1. Physiological parameters and mortality rates

For all three (vehicle group, 1 and the 3 mg/kg MB) groups, the baseline heart rate (350–450 bpm), arterial oxygen saturation (94–96%), end tidal expiratory CO₂ (35–45 mmHg) were within normal physiological ranges and were not statistically different from each other, except for the arterial oxygen saturation of the 3 mg/kg MB group which showed a transient and mild reduction at the 60 min time point (from 94.9 to 91.0%, $p=0.03$) and returned to pre-MB value after the 90 min time point. This was likely due to light absorption of MB, which interfered with pulse oximetry. The mortality rates of the vehicle, 1 and 3 mg/kg MB groups were 1 out of 12, 3 out of 12 and 2 out of 7 at 24 h after stroke, respectively.

2.2. Lesion volume evolutions

2.2.1. Overview

Representative CBF maps at 30 min, ADC maps at 30 and 180 min, and T2 maps at 24 h are shown in Fig. 1 for the vehicle, 1 and 3 mg/kg MB groups. At 30 min after MCAO, abnormal CBF and ADC were detected and a perfusion and diffusion mismatch was present. At 30 min after MCAO, the ADC lesion volumes after MCAO were similar for all three groups. At 180 min after MCAO, the ADC lesion volumes of the vehicle group were larger than those of the MB treated groups at 180 min. At 24 h after MCAO, T2 infarct volumes were similar amongst the groups.

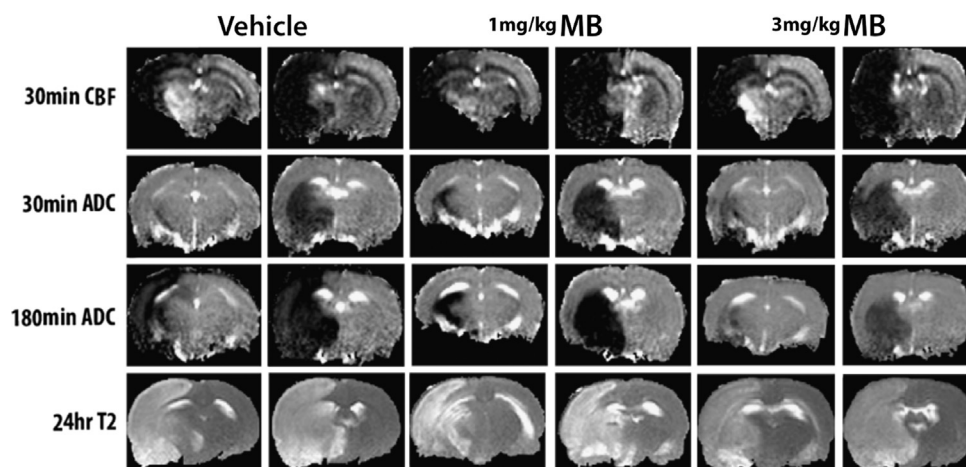


Fig. 1 – Representative baseline cerebral blood flow (CBF) and apparent diffusion coefficient (ADC) lesion volumes at 30 min, ADC volumes at 180 min and T2 volumes at 24 h for the vehicle, 1 and 3 mg/kg methylene blue (MB) groups.

2.2.2. 1 mg/kg Group

CBF defined lesion volumes for the vehicle and 1 mg/kg group ranged from 300 to 320 mm³, were not statistically different from each other, and did not change across time (Fig. 2). At 30 min, the ADC lesion volumes of the vehicle and 1 mg/kg MB group were not statistically different from each other and both were statistically different (smaller, $p < 0.05$) from the CBF lesion volumes. In the vehicle group, the ADC lesion volume increased with time, predominantly within the first 30 min. By contrast in the 1 mg/kg MB group, the ADC lesion volume increased at a slower rate and was smaller than that of the vehicle group at 180 min. A repeated measure ANOVA for the 60, 90, 120, 150 and 180 min ADC lesion volume time points for the 1 mg/kg group versus vehicle was 0.041. The lesion volume of the 1 mg/kg group at 60, 90 and 120 min time points were statistically different ($p = 0.009$, 0.012, 0.018, respectively) from the vehicle-treated animals.

2.2.3. 3 mg/kg group

CBF defined lesion volumes for 3 mg/kg group at 30 min were about 290–310 mm³ and were not statistically different from the other groups (Fig. 3). At 30 min, the ADC lesion volume of the 3 mg/kg MB group was not different from that of the vehicle treated group at 30 min. The CBF ischemic volumes at 60, 90, 120 and 150 min for the 3 mg/kg MB were smaller than those of the vehicle ($p = 0.01$ –0.03), indicative of increased CBF in the 3 mg/kg MB group. Similar to 1 mg/kg, the administration of 3 mg/kg MB decreased the growth rate of the ADC lesion volume over the following 30–180 min. The ADC lesion volumes at 60, 90 and 120 min were smaller compared to the control group ($p = 0.034$, 0.021, 0.047). A repeated measures ANOVA analysis for the 3 mg/kg group yielded a p -value of 0.165 for the 60–180 min time points and a p -value of 0.047 for only the 60, 90 and 120 min time points. Conversely, the comparison of the ADC and CBF values (60–180 min) for the 1 and 3 mg/kg groups was not statistically different ($p > 0.05$).

2.3. Mismatch evolutions

To further evaluate the MB treatment effects, we separately analyzed the central and peripheral image slices to the lesion (Fig. 4a) for the 1 mg/kg MB group. In the central slices, the

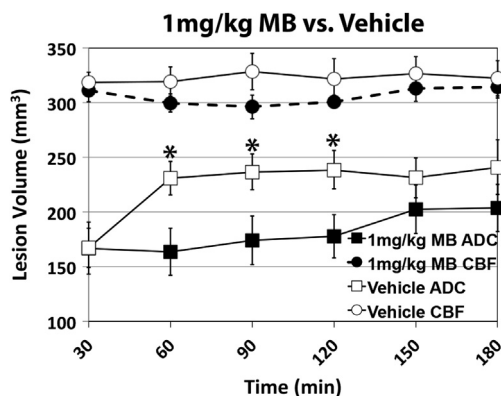


Fig. 2 – Temporal evolution of ADC and CBF lesion volumes for the 1 mg/kg MB and vehicle rats ($n = 12$ for each group). Circles (CBF) and squares (ADC) (* $p < 0.05$).

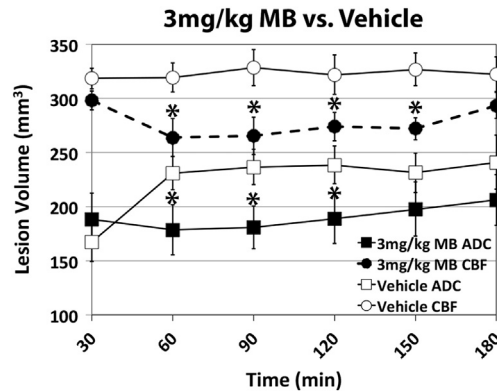


Fig. 3 – Temporal evolution of ADC and CBF lesion volumes for the 3 mg/kg groups ($n = 12$ for vehicle ADC and CBF, and $n = 5$ for 3 mg/kg CBF, and $n = 7$ for 3 mg/kg ADC). Circles (CBF) and squares (ADC) (* $p < 0.05$).

vehicle-treated animals demonstrated a rapid increase in ADC lesion volume, reaching CBF lesion volume 60 min after MCAO (Fig. 4b). However, the peripheral slices demonstrated delayed growth of the ADC lesion and there was persistent perfusion–diffusion mismatch at 180 min after MCAO (Fig. 4c). By comparison, the 1 mg/kg MB group showed decreased growth rate of the central and peripheral slices and overall small ADC lesion volumes up to 180 min after MCAO. The CBF volumes of the central and peripheral lesion also decreased for MB treated animals. The central CBF volumes for the 1 mg/kg MB versus vehicle groups at 60, 90, and 120 min were not different. Similar results were obtained from analysis of the 3 mg/kg MB group (data not shown).

2.4. 24 h T2 infarct volumes

The T2 infarct volumes at 24 h were 237 ± 9 , 235 ± 8 , and 229 ± 6 mm³, for vehicle, 1 and 3 mg/kg MB groups, respectively. They were not statistically different from each other as expected.

3. Discussion

We applied serial PWI, DWI and T2 MRI to test the hypothesis that MB administration can prolong the perfusion–diffusion mismatch. Our main findings are that: (i) MB significantly delays the progression of perfusion–diffusion mismatch into infarct, (ii) MB mildly increases the CBF in the hypoperfused tissue, (iii) MB does not change the final infarct volume in permanent ischemic stroke, and (iv) there are no dose-dependent effects on mismatch progression for the 1 and 3 mg/kg doses studied.

3.1. Safety

Similar to prior studies, low dose MB was found to be safe in rodents. The baseline heart rate, arterial oxygen saturation, end tidal expiratory CO₂ were within normal physiological ranges for all three groups. Low-dose MB (1–5 mg/kg) also has an excellent safety profile in humans, with minimal side

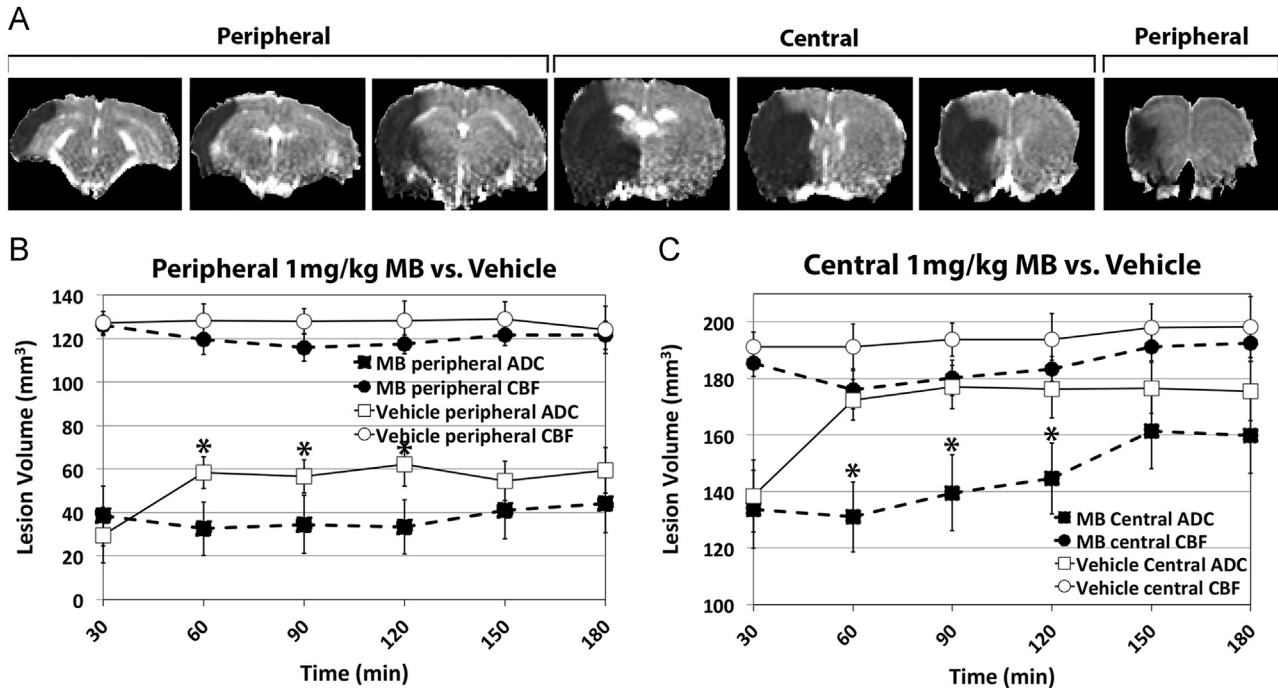


Fig. 4 – (a) Depiction of central and peripheral ADC volumes **(b)** temporal evolution of ADC and CBF lesion volumes in peripheral and **(c)** central slices ($n=12$ for each group) in the 1 mg/kg MB and vehicle-groups. Circles (CBF) and squares (ADC) (* $p<0.05$).

effects and contraindications (Peter et al., 2000; Walter-Sack et al., 2009). USP-grade MB is classified as a FDA-grandfathered drug that has been used in humans for over 120 years. The very few papers reporting negative effects can be readily explained. For example, one study (Wei et al., 1994) showed that ‘topical’ administration of MB given at the concentration of 1 mM inhibited guanylyl cyclase and thus reduces the second messenger cyclic GMP, but did not alter regional CBF and oxygen consumption in focal cerebral ischemia. However, this was an extremely high MB concentration (over 1000 times higher than MB concentrations with beneficial effects). MB has a hormetic dose-response, with therapeutic effects at low dose and toxic effects at high doses (Bruchey and Gonzalez-Lima, 2008; Callaway et al., 2004). Daily 4 mg/kg oral MB has been used safely for one year in clinical trials (Naylor et al., 1986; Wischik et al., 2008; Wischik and Staff, 2009). MB at 1–3 mg/kg i.v. infused over minutes is used safely as the treatment of choice for methemoglobinemia poisoning in emergency rooms.

3.2. MB metabolic and hemodynamic effects

There is evidence that MB has effects on metabolism in vitro, and on hemodynamic and metabolism in vivo. Lin et al. (2012) showed that low-dose MB increases the rate of cytochrome c activity when exposed to NADH in isolated rat brain mitochondria, and increases cellular oxygen production and quantitative cellular glucose uptake in HT-22 cells. Positron emission tomography studies using fluorodeoxyglucose (^{18}F -FDG) in rat demonstrated that MB increased neural glucose uptake in vivo. MRI-based neuroimaging studies in normal rats also showed that MB increased CBF, cerebral metabolic rate of oxygen consumption under normoxic and hypoxic

conditions. Additional neuroimaging studies also demonstrated that low dose MB enhanced the blood oxygenation level dependent (BOLD) fMRI responses elicited by forepaw stimulation (Huang et al., 2013).

3.3. MB delays mismatch progression

Low-dose MB administration delays perfusion–diffusion mismatch progression to infarction. The effect is more apparent in the peripheral slices compared to central slices (Fig. 4b and c), consistent with the notion that peripheral slices have more tissue at risk. A possible explanation is that MB enhances cytochrome c activity and sustains mitochondrial ATP production as well as prevents oxygen free radicals formation as described above.

Another potential mechanism behind the in vivo neuroprotective effect of MB may be related to an increase in perfusion. Our data showed that MB at 3 mg/kg transiently increased CBF in the hypoperfused tissue in the hyperacute phase. Lin et al. (2012) also reported similar effects under normoxia and hypoxia in normal animals. The 1 mg/kg MB administration also produced a mild increase in CBF that approached, but did not reach statistical significance ($p=0.07$).

3.4. Comparison with previous studies

Our results are consistent with two previous studies of MB treatment on transient ischemic stroke in rats (Shen et al., 2013; Wen et al., 2011). Wen et al. showed by histology that low dose MB given at 60 min after MCAO in a rat transient (60 min) MCA occlusion model decreased infarct volume at 24 h. The novelty of our study is that MRI was used to

longitudinally demonstrate that MB delayed the progression of the perfusion–diffusion mismatch infarct, which would not have been possible with terminal histological methods.

Shen et al. used neuroimaging in a 60 min rat MCA occlusion model to study MB. A pixel-by-pixel approach was used to assess the effects of MB on the mismatch tissue. MB was able to salvage more core pixels and mismatch pixels than vehicle treated animals, resulting in ~30% reduction of infarct volume at 2 days after stroke in the MB group compared to vehicle group. The novelty of our work includes testing the efficacy of MB in permanent ischemia where MB administration delayed perfusion–diffusion mismatch progression to infarction. This is clinically relevant because MB may be used to delay the evolution of infarct prior to chemical or mechanical thrombectomy.

3.5. Dose dependence

We also tested whether a higher dose MB can increase the efficacy of the drug in ischemic stroke. We were surprised to find that 3 mg/kg MB did not improve efficacy compared to 1 mg/kg. This may be due to the hormetic dose response curve of methylene blue which is relatively flat over the therapeutic dose ranges (Fig. 3 in Bruchey and Gonzalez-Lima, 2008). Future studies will aim at studying the efficacy of multiple MB doses to improve functional outcome in chronic stroke.

4. Conclusion

The use of low dose (1 and 3 mg/kg) methylene blue delays the growth rate of the perfusion–diffusion mismatch into infarction, thereby extending the treatment window. This neuroprotective effect is likely the result of sustained ATP production and increased CBF to tissue at risk. Since low dose methylene blue has a good safety profile in humans, this preclinical work could expedite its transition into clinical trials.

5. Experimental procedures

5.1. Animal preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center. Male Sprague-Dawley rats (250–350 g, $n=41$) were purchased from Charles River Laboratories (Wilmington, MA). Fourteen animals were allocated to vehicle, sixteen animals to 1 mg/kg MB, and eleven animals to 3 mg/kg MB. Permanent focal cerebral ischemia of the middle cerebral artery was performed using 0.35–0.37 mm intraluminal silicon rubber-coated filaments (Doccol Corporation, Sharon, MA) under 2% isoflurane. Animals were mechanically ventilated and maintained at 1.2–1.5% isoflurane in air while in the MRI scanner. A MouseOx system (Starr Life Sciences) was used to monitor heart rate, arterial oxygen saturation and breathing rate. A rectal probe sensor in conjunction with a water bath feedback loop was used to maintain the temperature within 37 ± 0.5 °C. End-tidal CO_2 was monitored using a SurgiVet V9004 Capnograph (Smiths Medical, Waukesha, WI). All

physiological measurements were recorded and maintained within normal range throughout the experiment.

5.2. Experimental procedures

A pre-determined randomized scheme was used to allocate the type of intervention prior to MCA occlusion surgery. The surgeon and data analyst were blinded to the type of intervention. Either vehicle (heparinized normal saline), 1 or 3 mg/kg USP methylene blue (10 mg/kg bottle, American Regent, Shirley, NY; diluted in heparinized normal saline) was infused through the tail vein over 30 min after initial baseline scan (at 30 min) using an MRI compatible pump (Harvard Apparatus). Half-dose (0.5 or 1.5 mg/kg) was administered after the last 180 min time point over 30 min. Standard behavioral tests were not obtained due to anticipated animal inactivity and very poor health associated with a permanent stroke model resulting in decreased sensitivity of these tests. Animals were excluded if the initial lesion volume at 30 min was less than 40 mm³ or greater than 300 mm³. This inclusion window minimized selection of lesions with poor occlusion or insufficient mismatch. Animals were also excluded if the perfusion imaging demonstrated baseline or delayed poor occlusion. Five animals were excluded due to initial lesion volume not meeting size criteria and two were excluded due to poor occlusion. Two additional animals were excluded due to abnormal physiology and another animal was excluded due to incomplete MRI data.

5.3. MRI experiments

MRI studies were conducted on a Bruker Biospec 7 T/40 cm scanner with a 76 G/cm BGA12S gradient insert (Billerica, MA) using custom made brain imaging and single loop perfusion neck coils. The apparent diffusion coefficient (ADC) was measured using spin-echo diffusion-weighted echo-planar imaging and two b values of 4 and 1200 s/mm², and TE=30 ms (Shen et al., 2005). The cerebral blood flow (CBF) was measured using continuous arterial spin labeling with a 2.7-s square radio-frequency pulse applied to the labeling coil followed by a 250 ms post-labeling delay and TE=10.2 ms. T2 maps were acquired using fast spin echo with four effective echo times (25, 40, 75 and 120 ms), echo train length 4, and 8 signal averages. The common parameters for these three sequences were: seven 1.5 mm thick slices, single shot, TR=3 s, matrix=96 × 96 (reconstructed to 128 × 128), field of view=25.6 × 25.6 mm, 90° flip angle. ADC and CBF maps were acquired at 30, 60, 90, 120, 150, and 180 min post-occlusion and on day-1 (24 ± 2 h) and T2 maps were acquired on day-1 (24 ± 2 h).

5.4. Data analysis

ADC and CBF measurements were collected every 30 min over 3 h after stroke to monitor the evolution of the ischemic penumbra (Meng et al., 2004). The 60 min time point was obtained immediately after termination of the drug infusion. T2 map was calculated at day 1. Cerebral blood flow measurements were used to estimate perfusion. In order to minimize the subjectivity associated with independent ROI measurements, ADC region of interest regions were drawn in each contralateral normal hemisphere including cortex and striatum

to correct for possible fluctuation in ADC signal. An upper limit signal threshold for infarcted ADC values was calculated from the mean normal hemisphere ADC signal minus three times the standard deviation of the non-infarcted tissue. Regions that met this 99.7% difference signal threshold in the contralateral (normal) hemisphere were not included in the infarct lesion volume measurements and were considered artifact. A threshold for abnormal CBF values was calculated from the mean normal hemisphere CBF signal minus one times the standard deviation of the non-infarcted hemisphere to be able to detect subtle changes in PWI volume. Final infarct volume was defined using T2 maps at day 1 (24 ± 2 h) using threshold of the mean T2 signal value of the normal hemisphere plus two times the standard deviation. Edema-corrected infarct volume was calculated as infarct volume – (right hemisphere volume – left hemisphere volume).

5.5. Statistical analysis

Lesions volumes are presented as mean \pm standard error of mean. ANOVA with repeated measures correction was used to evaluate global differences in lesion volumes across different 60–180 min time points. The Student's *t*-test was used to test the null hypothesis that the saline group mean lesion volume is not greater than the MB treated lesion volume for the individual time points. Statistical and data analysis was conducted in MedCalc 12.7.0 (MedCalc Software, Ostend, Belgium) and Stimulate 8.0.1 (University of Minnesota). Values were expressed as means \pm S.E.M. A *p* < 0.05 was considered statistically significant.

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