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Methylene blue treatment in experimental ischemic stroke: A mini-review

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Abstract:

Stroke is among the leading causes of death and long-term disability worldwide. Methylene blue (MB), a drug grandfathered by the Food and Drug Administration with a long history of safe usage in humans for treating methemoglobinemia and cyanide poisoning, has recently been shown to be neuroprotective in neurodegenerative diseases and brain injuries. The goal of this paper is to review studies on MB in experimental stroke models.

Key words:

Ischemia, methylene blue (MB), stroke

Introduction

Stroke is the second leading cause of death and the leading cause of long-term disability worldwide, and the fourth leading cause of death in the United States.^[1] There are 800,000 new or recurrent strokes per year in the United States. Of the 6 million Americans who are stroke survivors, 71% are unable to return to work. Over \$70 billion was expended on stroke patient care in 2013.^[1] This cost is steadily rising because the conditions that put people at the risk of stroke (such as heart disease, hypertension, diabetes, and obesity) are also steadily on the rise. Recombinant tissue plasminogen activator (rtPA), the only drug clinically approved to treat ischemic stroke, is limited to only a small subset of patients due to the serious risk of often fatal hemorrhagic transformation and can only be administered within 4.5 h of stroke onset.^[2] Recently, intraarterial therapy using primarily stent-retriever technology to achieve mechanical thrombectomy combined with intravenous (IV) rtPA administration has been found to be superior to IV rtPA alone when patients with proximal cerebral arterial occlusions are treated within 6 h of symptom onset.^[3] Despite the tremendous efforts taken in stroke research, our ability to minimize infarct volume and neurological deficit remains extremely limited. Thus, there is an urgent need to develop new treatments for stroke to protect the brain from the acute phase to the chronic phase.

In acute stroke, a therapeutic approach is to buy time (i.e., protecting neurons and glia *via* sustaining metabolic energy) before recanalization.^[4,5] This may allow the expansion of the critical treatment time window. During the reperfusion phase, it is important to minimize reperfusion injury such as that from excessive production of reactive oxygen species^[6,7] that could accelerate mitochondrial damage.^[8] During the chronic phase, the brain undergoes significant remodeling^[9,10] and it is important to maximize functional recovery. Thus, advanced drug or reagent methodologies, to enhance ischemic cells and tissues survival and assist the effect of thrombolytic treatment, are required in the development of effective therapies for the management of stroke patients. Mitochondrial targeting is one of the promising strategies that is widely explored.^[11,12]

Methylene blue (MB), a Food and Drug Administration (FDA)-grandfathered drug, is currently used to treat malaria, methemoglobinemia, and cyanide poisoning in humans.^[13,14] MB has been rigorously studied for over 120 years with 5,794 human MB studies listed in Pubmed (searched in December 2015). Low-dose MB (1-5 mg/kg IV) is very safe. Its pharmacokinetics, side effect profile, and contraindications are well-known and most importantly minimal in humans.^[15,16] There were only a few negative reports and they were associated with exceptionally high doses. For example, MB has been used in parathyroid

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surgery to aid in lymphatic mapping at doses of 3.5-10 mg/kg. The FDA also warned physicians about possible serious serotonin reactions in patients who received IV MB during parathyroid surgery if taking serotonergic psychiatric drugs. However, a subsequent report by Mayo Clinic surgeons and pharmacologists summarized the FDA evidence and literature and concluded "that the use of methylene blue dye at low doses for lymphatic mapping likely carries very little risk for serotonin neurotoxicity."^[17] There has never been any negative report based on oral MB. Daily 4 mg/kg oral MB has been used safely for 1 year in clinical trials.^[18] MB at 1-3 mg/kg IV is safely used as a standard treatment for metabolic poisoning in emergency rooms.

The mechanisms of action of MB are as followed. MB has renewable auto-oxidizing property, which acts as an electron cyler that allows MB to redirect electrons to the mitochondrial electron transport chain, thereby enhancing adenosine triphosphate (ATP) production and promoting cell survival. In bypassing complex I-III activity to generate ATP, MB reduces reactive oxygen species production from the mitochondrial electron transport chain. The antioxidant property of MB is thus unique. *In vitro* studies have firmly established that MB enhances cytochrome c oxidase (complex IV) activity to produce more ATP in cells under normoxia, and MB replaces oxygen as the oxidant to sustain ATP generation under hypoxia while simultaneously reducing oxidative stress.^[19-22] Moreover, chronic MB treatment also modifies mitochondrial function and induces long-lasting cellular changes.^[23] Specifically, repeated low-dose (0.5-2.0 mg/kg) MB has long-lasting upregulation of brain cytochrome c oxidase activity.^[20,24-26] MB readily crosses the blood-brain barrier because of its high lipophilicity.^[15]

Low-dose MB has recently been shown to reduce neurobehavioral impairment in optic neuropathy,^[19,27] traumatic brain injury,^[28] Parkinson's disease,^[23,29] Alzheimer disease,^[30-32] and ischemic stroke.^[4,5,33] The goal of this article is to review relevant MB literatures in relation to neuroprotection in experimental stroke models.

A Pubmed search (December 2015) resulted in 25 papers relevant to the use of MB in stroke or related to stroke [Table 1]. Our goal is to review pertinent findings from most of these.

Basic Stroke-related Methylene Blue Studies

One of the earliest MB experiments was performed by Sidi *et al.* in 1987.^[34] Arterial pressure transiently increased followed with MB (5 mg/kg) administration by using hemodynamic measurements in dogs. Wu and Bohr found the contraction produced by endothelin was augmented when the intact aortic rings were treated with MB (10^{-5} M) in aortas from Wistar-Kyoto rats but not in those from stroke-prone spontaneously hypertensive rats.^[37] Ishiyama *et al.* studied the inhibitory action of MB against nicorandil-induced vasodilation in dogs.^[40] Kontos and Wei demonstrated that MB could eliminate the arteriolar dilation in response to nitroprusside and nitroglycerin after permeabilization of the cell membrane.^[39] MB has been shown to increase blood pressure and myocardial function by inhibiting nitric oxide actions in human septic

shock disease.^[41,47,50,52] These studies demonstrated that MB has vascular effects and causes vasoconstriction transiently, thereby improving blood pressure, which could help to defend against hypoperfusion during stroke.

Nitric oxide generation during ischemia and reperfusion plays a significant role in ischemic and reperfusion injury.^[56] There is evidence that MB decreases or inhibits nitric oxide generation that might have the potential effect of neuroprotection in ischemia/reperfusion injury. In order to prove that the endocardial endothelium of *Rana esculenta* produces an amount of nitric oxide that is sufficient to modulate ventricular performance, Sys *et al.* measured the changes of stroke volume (as a measure of performance in paced frog hearts) and stroke work (as an index of systolic function) after using MB-induced inhibition of nitric oxide synthase.^[43] This reminded us that MB could inhibit nitric oxide generation. Evgenov *et al.* found that continuous infusion of MB counteracted the early myocardial dysfunction and derangement of hemodynamics and gas exchange by the inhibition of nitric oxide pathway in ovine endotoxemia model.^[48]

Xie *et al.* demonstrated that MB treatment activated 5'adenosine monophosphate-activated protein kinase signaling but did not inhibit mammalian target of rapamycin signaling in serum deprivation cells and normal mouse.^[57] This study suggests that MB-induced neuroprotection is mediated, at least in part, by macroautophagy. Additionally, MB treatment altered the levels of microtubule-associated protein light chain 3 type II, cathepsin D, Beclin-1, and p62, suggesting that it was a potent inducer of autophagy.^[58] Thus, MB may be related to autophagic cell death.

Ryou *et al.* studied the MB-induced neuroprotective mechanism focusing on stabilization and activation of hypoxia-inducible factor-1 α in an *in vitro* oxygen-glucose deprivation reoxygenation model.^[55] They found that MB activated the erythropoietin-signaling pathway with a corresponding increase in hypoxia-inducible factor-1 α and consequently related to apoptotic cell death. Together, these studies shed light on the molecular pathways that MB modulates.

Methylene Blue Studies in Ischemic Stroke

While low-dose MB has recently been shown to reduce neurobehavioral impairment in neurodegenerative diseases (*ca.* Parkinson's disease,^[23,29] Alzheimer's disease^[30-32]), the neuroprotective effects of MB on cerebral ischemia *in vivo* were only recently demonstrated. In 2006, a Swedish group found that IV MB at clinical dose was neuroprotective after experimental cardiac arrest in piglets using histology.^[59] Wen *et al.* showed that MB could significantly reduce focal cerebral ischemia reperfusion damage in a transient focal cerebral ischemia rodent model in 2011 using histology.^[60]

Di *et al.* demonstrated that MB improved neurological function, and reduced the infarct volume and the necrosis after acute cerebral ischemic injury by augmenting mitophagy.^[54] These improvements depended on the effect of MB on mitochondrial structure and function. Acute cerebral ischemia caused the disorder of and disintegration

Table 1: Published papers about MB related to stroke (searched in Pubmed in December 2015)

Year	Cell/animal	Dose	Function
1987	Dog	1-5 mg/kg	Increased arterial pressure transiently ^[34]
1988	Dog	10 ⁻⁵ M	Relaxation of middle cerebral arterial strips was attenuated ^[35]
1990	Human	10 ⁻⁵ M	Inhibited the relaxations induced by thrombin or bradykinin in human basilar arteries ^[36]
1990	Rat	10 ⁻⁵ M	Augmented the contraction produced by endothelin in intact aortic rings ^[37]
1991	Feline	10 ⁻⁵ M	Inhibited the magnesium deficiency-related dilations on the tone of middle cerebral arteries ^[38]
1993	Cat	5 mM	Eliminated the arteriolar dilation after permeabilization of the cell membrane ^[39]
1994	Dog	10 ⁻⁵ M	Inhibitory action of methylene blue against nicorandil-induced vasodilation in pial vessels ^[40]
1995	Human	2 mg/kg	Transiently and reproducibly increased arterial pressure associated with an improvement in cardiac function ^[41]
1996	Dog	5 mg/kg	Increased arterial pressure, pulmonary arterial pressure, and systemic and pulmonary vascular resistances but decreased cardiac index and regional blood flow ^[42]
1997	Frog	10 ⁻⁶ M	Inhibition of nitric oxide synthase ^[43]
1999	Rat	10 μM	Attenuated endothelium-dependent relaxation in the mesenteric artery ^[44]
1999	Human	4 mg/kg	Increases systemic vascular resistance and may improve myocardial function ^[45]
2001	Fish	10 ⁻⁶ M	Inhibited nitric oxide synthase ^[46]
2001	Human	2 mg/kg and 2 mg/kg/h for 1 h	Counteracted myocardial depression; maintained oxygen transport and reduced concurrent adrenergic support ^[47]
2001	Sheep	10 mg/kg and 2.5 mg/kg/h for 5 h	Counteracted the early myocardial dysfunction and derangement of hemodynamics and gas exchange by inhibiting the nitric oxide pathway ^[48]
2002	Rat	10 ⁻⁴ and 10 ⁻⁵ M	Attenuated endothelium-dependent relaxation in aorta ^[49]
2002	Human	3 mg/kg	Acute vasoconstrictive and positive inotropic effects during septic shock ^[50]
2005	Human	2 mg/kg	Preoperative methylene blue administration reduced the incidence and severity of vasoplegic syndrome ^[51]
2010	Human	1 mg/kg, 3 mg/kg, and 7 mg/kg	High dose of MB [§] enhanced splanchnic perfusion ^[52]
2012	HT22 cells	5 μM	Attenuated superoxide production and antioxidant ^[53]
2013	Rat	0.5 mg/kg and 1 mg/kg	MB treatment minimized ischemic brain injury and improved functional outcomes. ^[5]
2014	Rat	1 mg/kg and 3 mg/kg	MB delayed the growth rate of the perfusion-diffusion mismatch into infarction in permanent stroke models ^[4]
2015	Rat and PC12 cell	1 mg/kg, 5 mg/kg, or 10 mg/kg for rat and 0.5 μM for cell	MB promoted mitophagy by maintaining the MMP [§] at a relatively high level, which contributed to a decrease in necrosis and an improvement in neurological function, thereby protecting against acute cerebral ischemic injury ^[54]
2015	HT22 cells	1 μM and 10 μM	MB protects the hippocampus-derived neuronal cells against OGD [†] -reoxygenation injury by enhancing energy metabolism and increasing HIF-1α protein content accompanied by an activation of the EPO [†] signaling pathway. ^[55]
2015	Rat	1 mg/kg	MB induced neuroprotection by enhancing autophagy and reducing apoptosis in the perfusion-diffusion mismatch tissue following ischemic stroke ^[33]

*MB: Methylene blue, §MMP: Matrix metalloproteinase, †OGD: Oxygen-glucose deprivation, ‡EPO: Erythropoietin receptor

of mitochondrial structure while MB ameliorated the destruction of mitochondria. They also further revealed that the elevation of mitochondrial membrane potential by MB under oxygen-glucose deprivation conditions mediated the augmented mitophagy in an oxygen-glucose deprivation model *in vitro*.

Shen *et al.* evaluated the efficacy of MB to treat ischemic stroke in a transient middle cerebral artery occlusion model in rats using noninvasive multimodal magnetic resonance imaging (MRI).^[5] In a randomized double-blinded design in which vehicle or MB was administered after reperfusion, they found

that the initial lesion volumes defined by abnormal apparent diffusion coefficient ^[61] at 30 min after ischemia were not significantly different between the two groups. The final infarct volumes defined by T₂ changes 2 days after stroke increased in the vehicle group but decreased in the MB group, yielding a 30% difference in infarct volume [Figure 1]. Tracking tissue fate on a pixel-by-pixel basis showed that MB salvaged more initial ischemic core pixels compared to the control group, and more mismatch pixels compared to the control group. This study, for the first time, evaluated the efficacy of MB to treat ischemic stroke in rats using longitudinal MRI and behavioral measures.

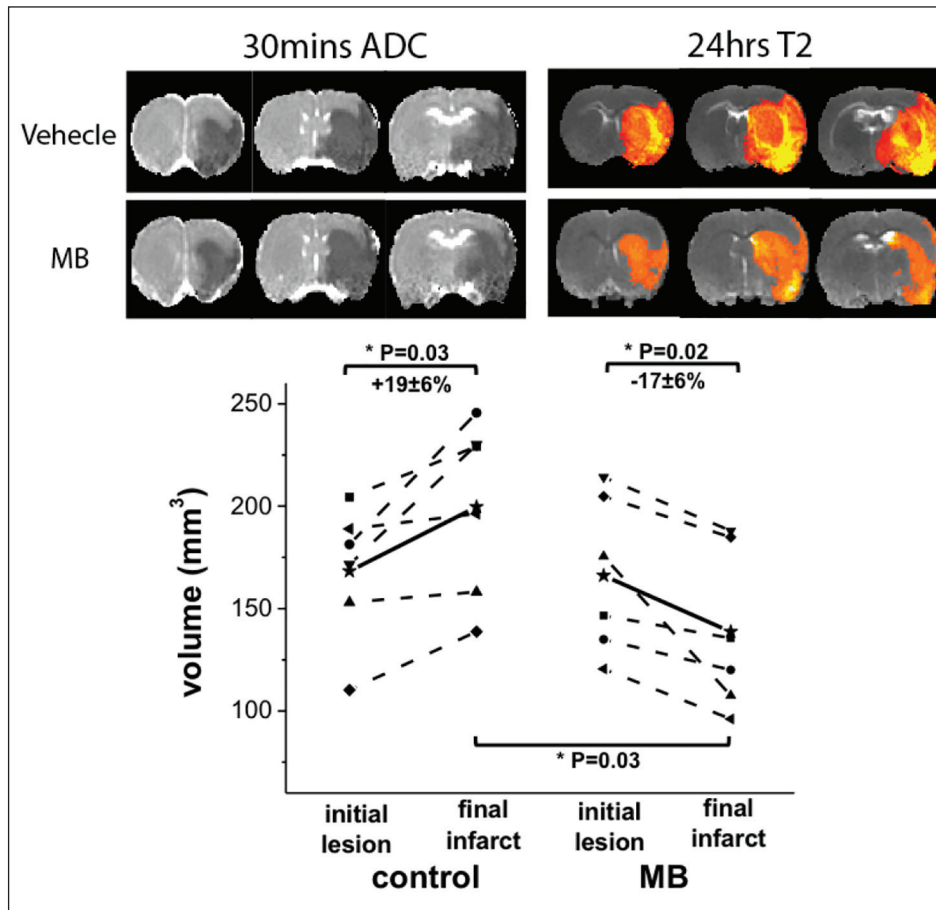


Figure 1: Initial lesion (30 mins ADC) and final infarct (24 hrs T2) volumes of vehicle- and MB-treated rats subjected to 60 min middle cerebral artery occlusion. Initial lesion and final infarct volumes of individual animal were connected using dot lines. Mean initial lesion and final infarct were connected using solid lines. Adapted from the reference paper⁶¹

Rodriguez *et al.* applied a similar multimodal MRI to test the hypothesis that MB 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.¹⁴¹ MB significantly prolonged the perfusion-diffusion mismatch, and mildly increased the cerebral blood flow in the hypoperfused tissue. MRI is now a routine neuroimaging tool in the clinic. MRI plays an important role in diagnosing, evaluating, and monitoring the cerebral tissue undergoing stroke and thereby, providing a noninvasive means to longitudinally evaluate treatment efficacy.

To further probe the underlying molecular mechanisms of neuroprotection of MB following transient ischemic stroke in rats, Jiang *et al.* employed noninvasive MRI to guide extraction of the different ischemic tissue types for western blot analysis of apoptotic and autophagic cascades.¹³³ Multimodal MRI during the acute phase and at 24 h were used to define three regions of interest (ROIs):

1. The perfusion-diffusion mismatch salvaged by reperfusion,
2. The perfusion-diffusion mismatch not salvaged by reperfusion, and
3. The ischemic core. The tissues from these ROIs were extracted for western blot analyses of autophagic and apoptotic markers.

The major findings were:

1. MB improved cerebral blood flow to the perfusion-diffusion mismatch tissue after reperfusion and minimized harmful hyperperfusion 24 h after stroke,
2. MB reduces infarct volume and behavioral deficits following transient ischemic stroke in rats,
3. MB improves cerebral blood flow (CBF) to at-risk tissue after reperfusion and minimizes harmful hyperperfusion 24 h after MCAO,
4. MB inhibits apoptosis and enhances autophagy in the at-risk tissue but not within the ischemic core,
5. MB modulates the p53-Bax-Bcl2-caspase3 cascade, inhibiting apoptotic signaling pathways,
6. MB modulates p53-AMPK-TSC2-mTOR cascades, enhancing autophagic signaling pathways [Figure 2].

Conclusion

Low-dose MB has a long history of safe usage in humans for treating methemoglobinemia and cyanide poisoning. MB also has energy-enhancing and antioxidant properties. There are substantial evidences that MB is neuroprotective for ischemic stroke. A number of studies have now investigated the mechanisms of action in ischemic stroke. Noninvasive MRI offers a means to identify neural correlates of neuroprotection, target specific tissue types for further investigation of molecular

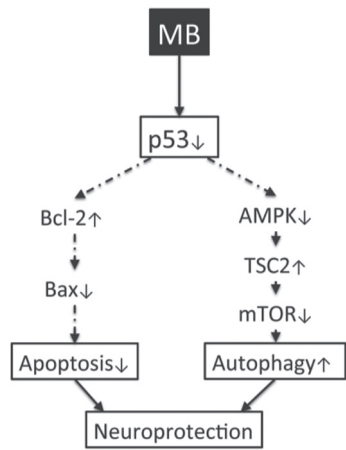


Figure 2: MB induces neuroprotection in neurons following cerebral ischemia through both the apoptotic p53-Bcl-2-Bax signaling pathway and autophagic p53-AMPK-TSC2-mTOR signaling pathway. The arrows show the effect of MB on these pathways. Adapted from the reference paper^{33]}

mechanisms of action, and longitudinally evaluate treatment efficacy. The excellent safety profile of low-dose MB in humans, together with noninvasive MRI, could expedite MB stroke clinical trials. MB treatments could offer novel therapeutic regimens in combination or alone to improve patient care following a stroke.

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Conflicts of interest

There are no conflicts of interest.

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