

Neuroprotective Effects of Methylene Blue on Scopolamine-Induced Amnesia via Anti-Cholinesterase and Anti-oxidative Activities in Adult Rat

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ABSTRACT

Methylene blue (MB) is currently used to treat methemoglobinemia, a blood disorder. But because high concentrations of methylene blue were known to damage the brain, no one thought to experiment with low concentrations. The study aimed to evaluate the possible protective effect of methylene blue against scopolamine induced- amnesia in adult rats. Methylene blue (2 and 5mg/kg, i.p.) was administered separately or concurrently with scopolamine administration (1mg/kg, i.p). Results showed that scopolamine induced amnesia in passive avoidance task, increased the catalytic activity of total cholinesterase and decreased content of acetylcholine depressed the level of reduced glutathione (GSH) and total antioxidant activity in the hippocampus and brain cortex. Methylene blue dose-dependently inhibited the enzymatic activity of total cholinesterase and increased the acetylcholine content, increased GSH content and total antioxidant activity in the hippocampus and brain cortex. Concurrent treatment of MB dose-dependently minimized both amnesic and anti-cholinergic effect of scopolamine. The study indicated that MB could protect against the scopolamine effects at low doses through its enhancing effect on cholinergic pathways and anti-oxidative activities. Methylene blue with its neuroprotective effects and could thus act as disease modifiers in patients, slowing the progression of behavioral deterioration since acetylcholinesterases themselves could contribute to the degenerative process.

Keywords: Alzheimer- scopolamine- methylene blue- cholinergic system- oxidative stress.

INTRODUCTION

Alzheimer's disease is a progressive, ultimately fatal, disorder in which certain types of nerve cells in particular areas of the brain degenerate and die for unknown reasons⁽¹⁾. However, it has been suggested that oxidative damage is one of the most integral neurotoxic mechanisms in both amyloid β ($A\beta$)

accumulation and tau pathologies^(2,3,4). Specifically, increased oxidative damage to brain lipids, carbohydrates, proteins and DNA has been reported to be involved in AD pathogenesis^(5,6). The vulnerable brain regions include the hippocampus and cortical area comprising cell populations involved in catecholaminergic, serotonergic and cholinergic neurotransmission⁽⁷⁻⁹⁾. The hippocampus is the processing

center for different information, experience and memory consolidation⁽¹⁰⁾. Various environmental stimuli, such as exploration, stress, or learning, increase acetylcholine (ACh) release to activate hippocampal functions⁽¹¹⁾. In addition, the drugs acting on the cholinergic system can improve or worsen cognitive abilities⁽¹²⁾. Consistently, the postmortem brain from Alzheimer's disease patients shows several indices of reduced cholinergic function, including deficits in the enzyme responsible for the synthesis of acetylcholine (ACh), choline acetyl-transferase, reduced ACh release, and loss of cholinergic neurons in the specific brain areas⁽⁴⁾.

Scopolamine is muscarinic receptor antagonist with amnesic property that has been used for decades in experimental animals to induce impairment in their performance of a variety of tasks requiring intact working and reference memory^(13,14). For many years, the amnesic action produced in animals by the administration of centrally acting muscarinic cholinergic antagonists, particularly scopolamine, has been a widely used model for the characterization of potential cognition-enhancing drugs⁽¹⁵⁾. However, the experimental models of memory impairment have been suggested to be of limited value because they fail to replicate the pathological aspects and the progressive degenerative nature of Alzheimer's disease⁽¹⁶⁾. Despite this limitation, scopolamine-induced memory impairment, particularly when coupled with a version of the inhibitory avoidance task provides a

relatively rapid phenotypic screening tool for drug discovery in the field of cognition enhancement.

Methylene blue (MB) is a diaminothiazine that has been in clinical use for approximately 100 years to treat a variety of ailments. MB treats congenital and poison-induced methemoglobinemia; prevents the side effects of chemotherapy^(17,18), and treats septic shock⁽¹⁹⁾. The dose of MB usually used in clinical settings is between 1 and 2 mg/kg/day⁽²⁰⁾; and signs of toxicity start at higher levels (7.5 mg/kg/day)⁽²¹⁾.

The therapeutic potential of MB also has been demonstrated in models for specific ailments. MB protects against endotoxin-induced lung injury, bacterial lipopolysaccharide-induced fever^(22, 23), and cyclosporine injury to the kidney⁽²⁴⁾. Methylene blue administration *in vivo* appears to benefit the central nervous system and cognitive function⁽²⁵⁾, protects from methylmalonate induced seizures⁽²⁶⁾, and protects from the cognitive decline inflicted by inhibitors of complex IV⁽²⁷⁾.

The present study aims to study the possible protective effect of MB in scopolamine-induced amnesia in rats and explore the neurochemical basis of this effect.

MATERIALS & METHODS

Experimental animals: male adult Sprague Dawley rats (150-200 g) were kindly provided from our breeding center at NODCAR and kept for a week for acclimatization under normal conditions and constant temperature (25±1C°) with ad libitum

water and food until starting the experiment.

Chemicals:

All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Scopolamine and methylene blue were dissolved in saline.

Animal grouping:

A total number of forty eight Sprague Dawley rats with an average weight of 175 ± 5 g, was divided into six groups:

- Control group: the rats intraperitoneally administered 1ml saline solution.
- Methylene group-2: the rats were intraperitoneally administered single dose of methylene blue (2mg/g).
- Methylene group-5: the rats were intraperitoneally administered single dose of methylene blue (5mg/g).
- Methylene group-2 plus scopolamine: the rats were intraperitoneally administered single dose of methylene blue (2 mg/g), followed by scopolamine 30 minutes later (1mg/kg, i.p).
- Methylene group-5 plus scopolamine: the rats were intraperitoneally administered single dose of methylene blue (5mg/g), followed by scopolamine 30 minutes later (1mg/kg, i.p).
- Scopolamine group: the rats were intraperitoneally administered single dose of scopolamine (1mg/kg).

Passive avoidance test

Passive avoidance test, which is a fear-motivated test classically used to assess the effect of different

treatments on memory ⁽²⁸⁾. The apparatus was equipped with identical illuminated and non-illuminated boxes with a guillotine door (5×5cm). The illuminated compartment (20×20×20cm) contained a 50-W bulb, and the floor of nonilluminated compartment (20×20×20cm) was composed of 2 mm stainless steel rods spaced 1 cm apart. A rat was gently placed in the illuminated compartment for an acquisition trial, and the door between the two compartments was opened after 10 sec. When the rat entered the dark compartment, the door was closed and an electrical foot shock (0.5 mA, 3 sec duration) was delivered through the stainless steel rods. Twenty-four hours after this acquisition trial, the mouse was again placed in the illuminated compartment for a retention trial. The time taken for a mouse to enter the dark compartment after door opening was defined as latency time for both acquisition and retention trials. Latency for entering the dark compartment was recorded up to 300 sec. If a rat did not enter the dark compartment within 300 sec., the rat was removed and assigned a latency score of 300 sec. Methylene blue and scopolamine were dissolved in 0.9% saline. Methylene blue doses were given 1 h after the acquisition trial. Memory impairment was induced in rat with scopolamine (1 mg/kg, i.p.) 30 min after methylene blue treatment. The control group received saline solution only. Following the behavioral study, the rats were decapitated and brain was dissected into cortex and hippocampus.

- Acetylcholine was determined according to HPLC⁽²⁹⁾. Total

cholinesterase activity was determined according to method of Ellman⁽³⁰⁾. Reduced glutathione levels were determined according to the Jayatilleke and Shaw⁽³¹⁾. Total antioxidant activity was determined using the colorimetric method of Blois⁽³²⁾.

Statistical Analysis.

Data presented as means \pm SE. One-way ANOVA followed by LSD test were used to evaluate significant differences from the control and SCP-treated groups. $P < 0.05$ was considered to be statistically significant. Statistical processor system support (SPSS) for Windows software, release 10.0 (SPSS, Inc, Chicago, IL) was used.

RESULTS

Data in tables 1, 2 and 3 show that scopolamine treatment

significantly increased ChE activity and decreased ACh content, and depressed total antioxidant activity and GSH level in brain cortex and hippocampus. Methylene blue treatment dose dependently decreased ChE activity and increased ACh content, total antioxidant activity and GSH content in brain cortex and hippocampus compared with control group (Table 1, 2 and 3). Whereas, MB pretreatment dose dependently normalized ChE activity and levels of ACh, GSH and total antioxidant activity in brain cortex and hippocampus (Tables 1, 2 and 3). In passive avoidance task, scopolamine administration decreased the retention time. Methylene blue treated rats exhibited higher retention time compared with control and scopolamine treated rats. Pretreatment of MB dose dependently attenuated the scopolamine effects (Fig.1).

Table (1): Effect of Methylene Blue (MB) Pre-treatment (2 and 5 mg/ kg, i.p) (MB-2 and MB-5, respectively) on Acetylcholine (ACh) Content and Total Cholinesterase (ChE) Activity in Brain Cortex of Scopolamine (SCP, 1mg/kg, i.p.) -Treated Rats.

Groups	ChE (U/g tissue wt)	ACh (μ g/g tissue wt)
Control	4040.50 \pm 74.70	1.63 \pm 0.08
MB-2	3240.83 \pm 60.31 ^{*,+}	1.82 \pm 0.06 ^{*,+}
MB-5	2941.50 \pm 22.69 ^{*,+}	2.27 \pm 0.06 ^{*,+}
MB-2+SCP	4428.17 \pm 338.60 ⁺	1.41 \pm 0.05 ^{*,+}
MB-5 +SCP	4588.33 \pm 175.44 ^{*,+}	1.60 \pm 0.03 ⁺
SCP	5888.33 \pm 242.15 [*]	0.74 \pm 0.06 [*]

Data presented as means \pm S.E. (n=8), $P < 0.05$ was considered to be statistically significant

^{*} Significant different from control group

⁺ Significant different from scopolamine treated rats

Table (2): Effect of Methylene Blue (MB) Pre-treatment (2 and 5 mg/ kg, i.p) (MB-2 and MB-5, respectively) on Acetylcholine (ACh) Content and Total cholinesterase (ChE) Activity in Brain Hippocampus of Scopolamine (SCP, 1 mg/kg, i.p.) - Treated Rats.

Groups	ChE (U/g tissue wt)	ACh (μ g/g tissue wt)
Control	3870.00 \pm 75.63	1.63 \pm 0.06
MB-2	3150.00 \pm 81.65 ^{*,+}	1.95 \pm 0.07 ^{*,+}
MB-5	2743.67 \pm 90.20 ^{*,+}	2.28 \pm 0.18 ^{*,+}
MB-2+SCP	4270.33 \pm 94.53 ^{*,+}	1.54 \pm 0.04 ⁺
MB-5+SCP	3727.33 \pm 101.95 ⁺	1.58 \pm 0.06 ⁺
SCP	6031.00 \pm 138.94 [*]	0.78 \pm 0.04 [*]

Data presented as means \pm S.E. (n=8), $P < 0.05$ was considered to be statistically significant

* Significant different from control group

⁺ Significant different from scopolamine treated rats

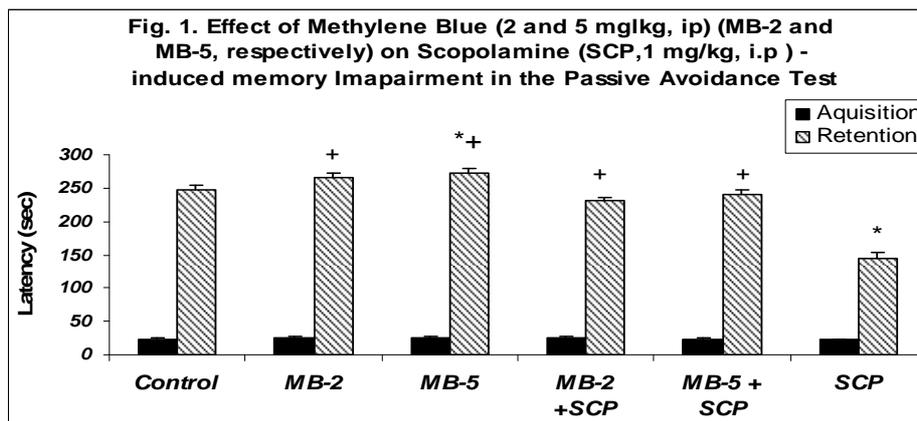
Table (3): Effect of Methylene Blue (MB) Pre-treatment (2 and 5 mg/ kg, i.p) (MB-2 and MB-5, respectively) on Total antioxidant activity and Reduced glutathione (GSH) Content in Brain Cortex and Hippocampus of Scopolamine (SCP, 1 mg/kg, i.p.) - Treated Rats.

Brain Areas	Groups	Total Antioxidant Activity (% inhibition of DPPH)	Reduced glutathione Ug/g
Cortex	Control	59.17 \pm 1.54	1.51 \pm 0.05
	MB-2	77.50 \pm 4.23 ^{*,+}	1.69 \pm 0.05 ^{*,+}
	MB-5	83.67 \pm 3.93 ^{*,+}	1.78 \pm 0.04 ^{*,+}
	MB-2+SCP	57.50 \pm 2.81 ⁺	1.44 \pm 0.05 ⁺
	MB-5+SCP	55.00 \pm 3.16 ⁺	1.55 \pm 0.04 ⁺
	SCP	40.00 \pm 2.24 [*]	1.17 \pm 0.04 [*]
Hippocampus	Control	60.83 \pm 5.54	1.61 \pm 0.05
	MB-2	73.00 \pm 3.65 ^{*,+}	1.77 \pm 0.04 ^{*,+}
	MB-5	78.00 \pm 2.82 ^{*,+}	1.89 \pm 0.03 ^{*,+}
	MB-2+SCO	55.17 \pm 2.89 ⁺	1.53 \pm 0.04 ⁺
	MB-5+SCO	62.67 \pm 2.19 ⁺	1.69 \pm 0.06 ⁺
	SCO	40.00 \pm 3.87 [*]	1.24 \pm 0.03 [*]

Data presented as means \pm S.E. (n=8), $P < 0.05$ was considered to be statistically significant

* Significant different from control group

⁺ Significant different from scopolamine treated rats.



Data presented as means \pm S.E. (n=8), $P < 0.05$ was considered to be statistically significant

* Significant different from control group

+ Significant different from scopolamine treated rats

DISCUSSION

The present study showed that scopolamine treated rats exhibited decreased level of ACh and increased catalytic activity of total cholinesterase in both brain cortex and hippocampus, due to the increased turn over of ACh as a result of ChE activation under scopolamine treatment. It might be speculated that scopolamine reversibly binds to some allosteric site in ChE molecule causing a change in its secondary structure leading to increased catalytic activity and decreased ACh content. In accordance, several studies indicated that scopolamine administration leads to the activation of AChE enzyme⁽³³⁻³⁵⁾. In addition, scopolamine induces oxidative stress possibly by impairing mitochondrial function, and/or AChE activation and subsequent ACh depletion. Consistently to this interpretation,

recent previous studies demonstrated the involvement of AChE activity in a cellular model of oxidative stress⁽³⁶⁾. In accordance, several studies linked the cholinergic dysfunction with the occurrence of oxidative stress^(37,38). Besides, both cholinergic dysfunction and oxidative stress play reciprocal roles in Alzheimer disease⁽³⁹⁾.

In the present study, methylene blue demonstrated an antioxidant effect and cholinergic neurotransmission potentiation through increasing GSH content and total antioxidant activity and inhibition of ChE activity and elevating ACh content in both brain cortex and hippocampus. In accordance, several studies indicated the antioxidant and the inhibitory effect of MB on ChE activity^(40,41).

In addition, MB pretreatment antagonized scopolamine- induced cholinergic dysfunction and oxidative stress, in a dose dependent manner.

This effect might be due to the antioxidant and augmenting effect MB on cholinergic transmission. Because its redox potential is close to zero, MB is very efficient in cycling between oxidized and reduced forms by suitable redox centers and reducing agents such as those in the mitochondria⁽⁴²⁾. MB is efficiently reduced by NAD (P) H dependent dehydrogenases to form the colorless MBH2⁽⁴²⁾. Electron delocalization in MB results in a partial positive charge located on both nitrogen and sulfur atoms, which may increase the permeability of MB through membranes. The lipid solubility of MBH2 is higher than for MB; thus, both forms enter the mitochondria⁽⁴³⁾. In accordance, several studies show that MB increases brain oxygen consumption, improves mitochondrial respiration and prevents free radical damage by serving as a redox compound at low doses and improves brain function^(25,44-47). In addition, methylene Blue (MB), is efficiently trapped in the brain and its concentration is over 10 times higher in the brain than in the circulation one hour after systemic administration⁽⁴⁸⁾, indicating a rapid and extensive accumulation in the nervous system. Moreover, MB has been used as a neuroprotective agent in drug-induced encephalopathy, dementia and manic-depressive psychosis⁽⁴⁹⁾.

In the present study scopolamine treated group showed defective performance in passive avoidance task, indicating the occurrence of the amnesia. This effect might be, at least, due to cholinergic dysfunction and/or oxidative stress. In accordance, previous studies indicated the

amnesic effect of scopolamine⁽⁵⁰⁻⁵²⁾. The observation that methylene blue treatment significantly activates memory and antagonized the amnesic effect of scopolamine is probably due to its activating effect of mitochondrial function and cholinergic up regulating effect in brain cortex and hippocampus. Consistent to this interpretation, several studies indicating the beneficial effect of MB with low doses on memory and brain function^(42,46), by enhancing the cholinergic neurotransmission^(12,53).

The study provides a demonstration of the neuroprotective effects of methylene blue in scopolamine-induced amnesia. Co-administration of MB, within the safe range (2-5 mg/kg, i.p.), provided effective protection against oxidative stress and cholinergic dysfunction, the main culprits in Alzheimer's disease. Although methylene blue merely presents a protective potential and may not cure Alzheimer's disease, yet any drug that reverses symptoms, improves quality of life, delays neurodegeneration, and saves huge costs, represents an important step in progress towards curing Alzheimer's disease.

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التأثيرات الوقائية لمادة الميثيلين الأزرق ضد فقدان الذاكرة الناجم عن حقن عقار سكوبولامين و ذلك عن طريق تثبيط انزيم كولين استيريز و منع الاجهاد التأكسدي في الجردان البالغة

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قسم الفسيولوجي- الهيئة القومية للرقابة و البحوث الدوائية

يستخدم الميثيلين الأزرق في علاج زيادة نسبة ميتهموجلوبين الدم، و لكن استخدام تركيزات عالية من الميثيلين الأزرق يؤدي إلى تلف الدماغ و أضرار فسيولوجية، تهدف الدراسة إلى تقييم التأثير الوقائي المحتمل للميثيلين الأزرق ضد فقدان الذاكرة الناجم من حقن عقار سكوبولامين في الجردان البالغة. ويتم حقن الجردان بمادة الميثيلين الأزرق بجرعة مقدارها ٢ و ٥ مجم/ كجم ثم يتم حقن عقار سكوبولامين في التجويف البريتوني بجرعة واحدة مقدارها ١ مجم. وأظهرت النتائج حقن عقار سكوبولامين يؤدي إلى فقدان الذاكرة في الاختبار السلوكي التجنب السلبي، وزيادة نشاط انزيم الكولين استيريز وانخفاض إجمالي محتوى أستيل الكولين وانخفاض مستوى الجلوتاثيون المختزل و المجموع الكلي لمضادات الأكسدة في قشرة الدماغ والحصين. أدت المعالجة بمادة الميثيلين الأزرق إلى تثبيط النشاط الانزيمي للكولين استيريز وزيادة المحتوى أستيل الكولين، وزيادة محتوى الجلوتاثيون و المجموع الكلي لمضادات الأكسدة في قشرة الدماغ والحصين. كما أدت المعالجة بمادة الميثيلين الأزرق إلى منع تأثير عقار سكوبولامين في كل من فقد الذاكرة والاجهاد التأكسدي و المحور الكوليني. وأشارت الدراسة إلى أن الميثيلين الأزرق في الجرعات المنخفضة يمكن أن يحمي من آثار سكوبولامين و ذلك من خلال تأثيره على تعزيز المسار الكوليني والنشاط المضادة للأكسدة. الأزرق ويمكن أن يساعد في ابطاء تطور تدهور الحالة الذهنية لدى المرضى بمرض ألزهايمر Alzheimer's