Neurological Mechanisms of Action and Benefits of Methylene Blue

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Abstract

Methylene blue (MB), a synthetic basic dye, is widely used as a stain and indicator in biology and chemistry. In addition to its traditional applications, MB has gained recognition for its medical uses, serving as an antidote for poisoning and being explored as a potential treatment for conditions like malaria, Alzheimer's disease, and cancer. Of particular interest is its application in neurology, notably in the management of neurodegenerative disease and traumatic brain injury (TBI). This paper provides a concise review of the neurological benefits of MB, emphasizing its mechanisms of action. By examining pertinent research, it highlights the potential of MB as a therapeutic agent in neurology, with implications for the development of new treatment strategies for neurological disorders and injuries.

Introduction

Methylene blue (MB) is a synthetic basic dye that belongs to the thiazine class of dyes. It has a deep blue color and is commonly used as a biological stain and chemical indicator in various applications. In biology, methylene blue is used to stain cells and tissues for microscopic observation. It can be used to identify certain structures within cells and to distinguish between different cell types. MB is also used in microbiology to differentiate between different types of bacteria based on their staining properties. In chemistry, methylene blue is used as an indicator in redox titrations. It changes color from blue to colorless when it is reduced, making it useful for determining the endpoint of a biological reaction. MB also has a range of medical uses. It can be used as an antidote for certain types of poisoning, including cyanide and methemoglobinemia. In addition, MB has been studied as a potential treatment for various medical conditions, including malaria, Alzheimer's disease, and cancer. MB was first synthesized in 1876 by Heinric Caro, a German chemist (Lee & Han, 2021). During this time, MB was used as an aniline dye for textiles. It was, however, found around that time that MB could be used in staining cells by binding their structure. The discovery served as a preparation ground for the application of MB in biological and medical studies. MB has also been widely applied in neurology in several ways including in treatment of traumatic brain injury (TBI). The purpose of this paper is to discuss the benefits of MB with a key focus on the neurological benefits and mechanisms of action from a neurological perspective.

Methylene Blue as a Neuroprotective Agent

MB has shown potential neuroprotective effects in several cytotoxicity-related diseases, including stroke, Parkinson's disease, and Alzheimer's disease. Its mechanism of action involves multiple beneficial

effects on cellular processes in the brain. One of the key properties of MB is its ability to act as an electron carrier (Rojas et al., 2012). This feature allows it to function against conditions such as malaria and methemoglobinemia. In cytotoxic situations in the brain, MB promotes cellular oxygen consumption and reduces anaerobic glycolysis. By doing so, it can help maintain adequate energy production in neurons and protect them from damage. Also, studies have shown that MB can decrease the production of reactive oxygen species (ROS) (Aldas, 2019). ROS are highly reactive molecules that can cause cellular damage and contribute to neurodegenerative diseases. By reducing ROS levels, MB helps protect cells from oxidative stress and glutamate toxicity, which is implicated in conditions like Alzheimer's disease.

Furthermore, it has been shown that MB can attenuate the decrease in mitochondrial membrane potential, which is crucial for maintaining mitochondrial function. It can also protect against certain neurotoxicity, such as that induced by IAA (indole 3-acetic acid). In comparison to antipsychotic drugs like phenothiazine, MB exhibits similar neuroprotective effects but operates through a different mechanism (Aldas, 2019). While phenothiazine, and several similar drugs act as a free radical scavenger independently of the mitochondria, methylene blue functions as a mitochondria-specific antioxidant. Its targeted action on mitochondria may contribute to its effectiveness in preserving cellular function and mitigating damage in neurodegenerative diseases. Additionally, MB has shown promise in preventing and managing brain damage associated with tumors. Certain alkylating agents used in tumor treatment, such as ifosfamide, can cause significant brain damage. MB appears to play a role in the oxidation of NADH and the restoration of mitochondrial enzymes, which may contribute to its ability to mitigate brain damage in these cases. However, the precise mechanisms underlying this protective effect are not yet fully understood.

MB Applications in Pain Lessening

Methylene blue has been studied for its potential anti-inflammatory and analgesic properties. A study by Zhang et al. (2016) shows that inflammation is closely associated with pain and can contribute to the development and progression of pain-related conditions. Moreover, studies such as Harth and Nielson, 2019 and Matsuda et al., 2019 have explored the relationship between inflammation and nociceptive (pain) responses. The findings from these studies have revealed that MB helps in reducing the formation of nitric oxide (NO) by directly suppressing the expression of endothelial nitric oxide synthase (eNOS). NO is a signaling molecule involved in various physiological processes, including vasodilation and inflammation.

Matsuda et al. (2019) has offered that MB also inhibits the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). This inhibition occurs by suppressing the expression of soluble guanylate cyclase (sGC), an enzyme responsible for the production of cGMP. cGMP is involved in numerous cellular signaling pathways, including those related to vasodilation. Additionally, the combined effects of MB on eNOS and sGC expression result in vasoconstriction, which means the narrowing of blood vessels. Vasoconstriction can impact blood flow and may have implications for various physiological processes, including inflammation and pain (Pan et al., 2019).

In the context of tissue injury, Studies have found out that iNOS (inducible nitric oxide synthase) functions as a strong inflammatory mediator in different types of cells (Pan et al., 2019). It inhibits Sirt1 activation through NO-mediated S-nitrosylation. This inhibition, in turn, activates NF-κB and p53, facilitating the expression of inflammatory

cytokines and apoptosis, respectively (Zhang et al., 2016). NF-κB activation intensifies these events by activating the iNOS/NO-NF-κB pathway. MB directly reduces iNOS expression and interferes with the binding of NF-κB to the iNOS promoter, thus interrupting this inflammatory signaling. During nerve injury, NMDA receptors are activated, leading to calcium influx. This excessive calcium influx results in the increased expression of nNOS (neuronal nitric oxide synthase) and activation of the nNOS/NO signaling pathway (Pan et al., 2019). The elevated production of NO stimulates NMDA receptors, triggering the NO/cGMP/PKG cascade. This cascade promotes the upregulation of BDNF (brain-derived neurotrophic factor) and neurotransmitter release, ultimately inducing long-term hyperexcitability and central sensitization. BDNF and peroxynitrite further potentiate NMDA receptors, which stimulate nNOS expression again. However, MB weakens these responses by inhibiting nNOS and sGC activation, potentially preventing the development of persistent pain.

In his study, Nakazawa et al. (2017) evaluated the effects of MB on iNOS/NO -NF-κB and found that MB (methylene blue) weakens inflammation by inhibiting the iNOS/NO pathway, which is involved in the pathogenesis of inflammation as a pro-inflammatory mediator. The iNOS/NO pathway activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and tumor protein p53 (p53), two transcription factors that play important roles in inflammation and cell death, respectively (Nakazawa et al., 2017). Silent information regulator 1 (Sirt1), a deacetylase, has a protective role under stress conditions and inactivates NF-κB and p53 by deacetylating them. However, the iNOS/NO pathway can inactivate Sirt1 through S-nitrosylation, thereby activating NF-κB and p53 (Zhang et al., 2016). NF-κB activation promotes the expression of inflammatory cytokines and upregulates iNOS, further enhancing inflammatory signaling. On the other hand, p53 activation induces cell

death. Therefore, MB directly inhibits iNOS expression and also by attenuating the binding of NF-κB to the iNOS promoter. As a result, the iNOS/NO-NF-κB signaling pathway is blocked, preventing the activation of NF-κB, the expression of inflammatory cytokines, and the subsequent detrimental effects of inflammation (Nakazawa et al., 2016).

In a study by Cohen et al. (2000), '*Reduced NO Accumulation in Arthrotic Cartilage by Exposure to Methylene Blue*' it was found that in human cartilage explants, MB demonstrated a dose-dependent decrease in nitric oxide (NO) accumulation and inducible nitric oxide synthase (iNOS) expression. These findings suggest that MB can effectively inhibit the iNOS/NO pathway, which is involved in cartilage degradation. Additionally, the study showed that MB upregulated transforming growth factor beta (TGF-β) receptors, which are crucial for cartilage matrix synthesis. By promoting TGF-β receptor expression, MB may contribute to the preservation of cartilage matrix and proteoglycan, ultimately preventing cartilage degradation (Cohen et al., 2000). Another study seeking to demonstrate the effect of MB on inhibiting the inflammatory process of the acetic acid-induced colitis in the rat colonic mucosa, the findings indicated that MB significantly reduced NO production and levels of inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Dinc et al., 2015). This finding is an indication that MB has anti-inflammatory properties in the context of ulcerative colitis. Moreover, the application of MB resulted in tissue injury and edema reduction in the submucosa, leading to a notable improvement in intestinal permeability. These findings suggest that MB can mitigate tissue damage and inflammation, thereby enhancing the functional state of the inflamed intestinal tissues (Dinc et al., 2015).

Yadav and Surolia (2019) propose that during nerve injury, Toll-like receptor 4 (TLR4) plays a crucial role in initiating neuroimmune activation in the nervous system. TLR4 activation leads to the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which promotes the expression of pro-inflammatory cytokines and contributes to the development of inflammatory hyperalgesia, a condition characterized by increased sensitivity to pain (Yadav and Surolia, 2019). Studies have shown that TLR4 activation via the TLR4/NF-κB pathway is a critical factor in the pathogenesis of neuropathic and chronic pain. Therefore, targeting the TLR4/NF-κB pathway has been proposed as a potential therapeutic strategy for relieving nerve injury-induced neuroinflammation and pain. By inhibiting TLR4 activation and subsequent NF-κB activation, it may be possible to attenuate the inflammatory response and alleviate pain associated with nerve injury. Deng et al. (2015) found that TLR4 has been found to be involved in the activation of inducible nitric oxide synthase (iNOS), which plays a role in the production of nitric oxide (NO). Given that MB has been shown to downregulate iNOS expression and inhibit NF-κB activation, it is possible that MB may be involved in suppressing the development and maintenance of neuropathic pain. By blocking the iNOS/NO pathway and attenuating NF-κB activation, MB could potentially mitigate neuroinflammation and pain associated with nerve injury (Deng et al., 2015). A clinical study demonstrated that systemic administration of MB improved chronic refractory neuropathic pain in 10 patients. It is hypothesized that this improvement may be attributed to the blockade of the iNOS/NO pathway mediated by MB (Miclescu et al., 2015).

MB Administration During Traumatic Brain Injury

Cruz-Haces et al. (2017) refers to traumatic brain injury (TBI) as a Traumatic brain injury (TBI) is a condition characterized by damage to the brain resulting from an external force or trauma to the head. It is a significant health concern due to its potential for severe medical, social, and economic consequences. The effects of TBI can vary widely, depending on the extent and location of the injury, and can range from mild concussions to severe and long-lasting impairments. One important aspect associated with TBI is the increased risk factor it poses for the development of neurodegenerative diseases (Dewan et al., 2018). Research has shown that individuals who have experienced a TBI, especially severe or repeated injuries, may have an elevated risk of developing conditions such as Alzheimer's disease, Parkinson's disease, and chronic traumatic encephalopathy (CTE) (Dewan et al., 2018). The exact mechanisms underlying this increased risk are still being investigated, but it is believed to involve various processes, including chronic inflammation, accumulation of abnormal proteins (such as *tau*), and disruption of cellular processes in the brain. The long-term consequences of TBI, including the potential for neurodegenerative diseases, make it essential to understand and effectively manage this condition. Prevention strategies, early diagnosis, proper medical care, and rehabilitation programs play critical roles in minimizing the impact of TBI and optimizing the recovery process for individuals affected by it.

In the context of TBI, several studies have demonstrated the potential of MB as a neuroprotective agent. For instance, in a rat model of TBI, MB was shown to improve cognitive function and decrease brain edema, oxidative stress, and neuroinflammation (Liu et al., 2017).

In another study, MB administration following TBI was found to reduce neuronal cell death, improve mitochondrial function, and decrease inflammation and oxidative stress (Zhou et al., 2019). Additionally, a recent study showed that MB treatment can reduce zinc toxicity-induced neuronal

damage in a TBI rat model (Genrikhs et al., 2021). The studies mentioned demonstrate that MB shows effectiveness in reducing neurodegeneration and improving behavioral disorders in animal models of various neurodegenerative diseases, including stroke, global cerebral ischemia, Alzheimer's disease, Parkinson's disease, and TBI. This makes MB a promising candidate for further research and potential therapeutic use (Tucker et al., 2018). However, despite its efficacy, the exact mechanisms underlying MB's protective actions are not yet fully understood.

The neuroprotective properties of MB may be attributed to its ability to enhance autophagy, reduce brain edema, inhibit microglia activation, and reduce inflammation and zinc toxicity (Fenn et al., 2015). Additionally, as a tricyclic phenothiazine, MB can act as a redox catalyst in mitochondria, which are known to be therapeutic targets following brain injury (Yonutas et al., 2016). In the specific context of TBI, treatment with MB has been shown to improve functional outcomes, reduce neuroinflammation, and decrease the expression of S100 protein (a marker of brain injury) and blood-brain barrier permeability. These findings suggest that MB holds promise as a pharmacological compound with neuroprotective properties for the treatment of TBI. Therefore, MB shows potential as a therapeutic agent for many neurodegenerative diseases. (Yonutas et al., 2016). While its exact mechanisms of action are not fully understood, MB's ability to modulate various pathological processes and its neuroprotective effects make it an object of ongoing research and a promising candidate for further investigation and potential clinical applications.

MB and Neural Apoptosis After Traumatic Brain Injury

MB has been shown to improve mitochondrial function by acting as an alternative electron carrier. It bypasses blockages in complex I/III of the electron transport chain and efficiently transfers electrons from NADH to cytochrome c (cyt c). This process helps reduce electron leakage, enhance ATP production, and decrease the overproduction of reactive oxygen species (ROS). In animal models of AD, PD, ischemic stroke, and TBI, MB has been found to attenuate pathological and neurobehavioral impairments (Zhu et al., 2018). After TBI, MB treatment has shown promising effects in reducing neuroinflammation, lesion volume, and neurological damage. According to Zhu et al. (2018) mitochondrial dysfunction plays a crucial role in mediating various cascades that lead to secondary brain damage. The dysfunction of mitochondria in TBI is associated with several detrimental processes, including calcium overload, cellular excitotoxicity, the release of reactive oxygen species (ROS), and cell apoptosis (programmed cell death). Mitochondria are responsible for oxidative phosphorylation and ATP generation, which are vital for providing energy to repair damaged cells (Zhu et al., 2018). When mitochondria become dysfunctional, the production of ATP is reduced, which can further worsen cell damage. Given the significant role of mitochondrial dysfunction in the pathological processes of secondary brain damage following TBI, targeting mitochondria for treatment may hold potential in improving TBI prognosis.

In a study by Shen et al. (2019), the researchers conducted experiments to investigate the effect of Methylene Blue (MB) on mitochondrial function using an in vitro model of oxygen-glucose deprivation (OGD) in PC-12 cells. The purpose was to examine whether MB treatment could alleviate mitochondrial dysfunction caused by OGD injury. The results of the study showed that MB treatment had positive effects on mitochondrial function in vitro. It reduced the production of reactive oxygen species (ROS) in neurons, stabilized the neuronal mitochondrial membrane potential

(MMP), and increased adenosine triphosphate (ATP) production (Shen et al., 2019). These findings suggest that MB treatment has the potential to reverse the mitochondrial dysfunction induced by OGD injury. However, the study did not assess mitochondrial function directly in neurons present in tissue samples from the TBI + saline group and the TBI + MB group. The difficulty lies in distinguishing mitochondria specifically in neurons from other cell types in the injured brain tissue. As a result, the researchers were unable to directly determine the effect of MB treatment on mitochondrial function in neurons in the context of traumatic brain injury (TBI).

Shen et al. (2019) also found that administering Methylene Blue (MB) to mice with traumatic brain injury (TBI) had mitigating effects on neuronal apoptosis (programmed cell death) and blood-brain barrier (BBB) permeability. Mitochondria play a crucial role in the process of neuronal apoptosis following TBI. In cases of ischemia and hypoxia injuries, cytochrome c is released from the mitochondrial membrane. It binds with ATP and apoptotic protease activating factors (Apaf-1) to form apoptosome complexes. These complexes cleave procaspases, leading to the activation of caspase 3, which triggers neuronal apoptosis (Ola et al., 2011). Arimoto-Matsuzaki et al. (2016) provides that the release of reactive oxygen species (ROS) and the decrease in ATP production also contribute to neuronal apoptosis in the context of TBI. MB, by acting as an alternative electron carrier, can transfer electrons from NADH to cytochrome c, thereby increasing the stability of cytochrome c. This process enhances ATP generation and reduces the release of cytochrome c and the production of ROS (Wen et al., 2011). Consequently, MB treatment has the potential to reduce neuronal apoptosis following TBI.

The blood-brain barrier (BBB) consists of various components such as pericytes, astrocytes, endothelial cells, and tight junction proteins. Following traumatic brain injury (TBI), the release of reactive oxygen species (ROS) can directly downregulate the expression of tight junction proteins like ZO-1, contributing to increased BBB permeability. Additionally, astrocytes have the ability to transfer their mitochondria to damaged neurons as a rescue mechanism (Tally Watts et al., 2014). These processes, along with neuronal apoptosis, can lead to BBB damage and increased permeability. MB treatment has been shown to reverse these processes and help maintain BBB integrity. It has been demonstrated that MB treatment can attenuate inflammation in TBI by increasing the expression of anti-inflammatory cytokine IL-10 and reducing the expression of pro-inflammatory cytokine IL-1β in microglia, thereby reducing inflammatory-mediated BBB damage (Zhao et al., 2016). In animal models of TBI, MB treatment has shown positive effects not only on cognitive and motor function but also on overall neurological function. Previous studies have shown that MB can minimize neuronal degeneration, behavioral deficits, and lesion volume in TBI animals. Other research has indicated that MB exerts a neuroprotective effect in TBI by inhibiting microglial activation, reducing brain edema, and promoting autophagy.

While previous studies focused on the anti-inflammatory effects of MB in TBI, Shen et al. (2019) specifically investigates the impact of MB on BBB integrity and neuronal apoptosis, making it distinct. Considering the cumulative evidence, MB exhibits three main functions in TBI: anti-inflammatory effects, improvement of BBB integrity, and reduction of neuronal apoptosis. This highlights the potential of MB as a drug for improving the prognosis of TBI. Apart from its effects on BBB integrity and neuronal apoptosis, mitochondrial dysfunction following TBI is also implicated in other secondary damages. Synaptic mitochondria are crucial

for maintaining synaptic plasticity and normal neurotransmission. Dysfunction of synaptic mitochondria can contribute to neurodegeneration. Furthermore, studies have shown that synaptic mitochondria are more susceptible to injury in TBI compared to non-synaptic mitochondria, suggesting that MB may improve spatial memory in TBI mice partly by improving synaptic mitochondrial function. Additionally, mitochondria can interact with microRNAs (miRNAs) involved in cellular responses to TBI, indicating that MB might also regulate miRNAs in the context of TBI.

Kulber et al. (2016) provides that Mitochondrial dysfunction following traumatic brain injury (TBI) contributes to various secondary damages, including synaptic dysfunction, neurodegeneration, and impaired neurotransmission. Synaptic mitochondria, which are crucial for maintaining synaptic plasticity and normal neurotransmission, have been found to be more susceptible to injury compared to non-synaptic mitochondria in TBI models. This highlights the importance of synaptic mitochondrial function in TBI-related cognitive impairments. MB treatment has been shown to improve spatial memory in TBI mice, potentially by enhancing synaptic mitochondrial function. Furthermore, mitochondria can interact with microRNAs (miRNAs) that play a role in cellular cascade responses to TBI. This suggests that MB may have the ability to regulate miRNAs involved in TBI pathophysiology. Repurposing existing drugs, such as MB, is a recommended approach in modern pharmacology (Wang et al., 2017). MB has a long history of clinical use spanning almost 130 years, and its safety profile and side effects are well-established. Compared to developing new drugs specifically for TBI therapy, repurposing MB offers the advantages of cost and time savings, as well as the potential for rapid clinical application. However, while MB has demonstrated positive effects on the prognosis of TBI in animal models, the existing research findings are not sufficient to support immediate clinical use of MB in TBI patients (Tucker et al., 2018).

Further investigation is necessary to explore the full range of therapeutic effects of MB in secondary brain injury. This would help establish a stronger scientific basis for its potential clinical application in TBI treatment.

Conclusion

Methylene blue holds potential as a neuroprotective agent in various cytotoxicity-related diseases, including neurodegenerative disorders and conditions involving brain damage. Ongoing research aims to further elucidate its mechanisms of action and explore its therapeutic applications. MB also shows potential as a therapeutic agent for neurodegenerative diseases, including TBI. While its exact mechanisms of action are not fully understood, MB's ability to modulate various pathological processes and its neuroprotective effects make it an object of ongoing research and a promising candidate for further investigation and potential clinical applications.

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