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REVIEW
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Methylene Blue Application to Lessen Pain: Its Analgesic Effect and Mechanism

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Methylene blue (MB) is a cationic thiazine dye, widely used as a biological stain and chemical indicator. Growing evidence have revealed that MB functions to restore abnormal vasodilation and notably it is implicated even in pain relief. Physicians began to inject MB into degenerated disks to relieve pain in patients with chronic discogenic low back pain (CDLBP), and some of them achieved remarkable outcomes. For osteoarthritis and colitis, MB abates inflammation by suppressing nitric oxide production, and ultimately relieves pain. However, despite this clinical efficacy, MB has not attracted much public attention in terms of pain relief. Accordingly, this review focuses on how MB lessens pain, noting three major actions of this dye: anti-inflammation, sodium current reduction, and denervation. Moreover, we showed controversies over the efficacy of MB on CDLBP and raised also toxicity issues to look into the limitation of MB application. This analysis is the first attempt to illustrate its analgesic effects, which may offer a novel insight into MB as a pain-relief dye.

Keywords: methylene blue, pain, anti-inflammation, sodium current, denervation

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INTRODUCTION

In 1876, German chemist Heinrich Caro synthesized methylene blue (MB) for the first time in history, which was basically applied for textiles as an aniline dye. Around the same time, it was found that MB is capable of staining cells by binding to their structures, in addition, sometimes inactivating bacteria. This discovery prepared the innovative ground for biological or medical studies related to MB. Numerous scientists applied it to a variety of animal and bacterial studies, importantly Paul Ehrlich introduced it to humans in 1891 as an anti-malarial agent. Indeed, this dye has been introduced to treat different diseases even including dementia, cancer, and depression (Wainwright and Crossley, 2002; Schirmer et al., 2003; Schirmer et al., 2011).

In the present day, MB is primarily known for a vasoconstrictor. It downregulates basically nitric oxide (NO), which is responsible for relaxing vascular smooth muscle, and leads to vasoconstriction (Wolin et al., 1990; Pan et al., 2019). However, under pathological conditions, NO is overexpressed and then contributes to inflammation as a pro-inflammatory mediator (Luo and Cizkova, 2000; Lundberg et al., 2008; Leiper and Nandi, 2011). Of note, MB suppresses the iNOS/NO-mediated inflammatory signaling by directly downregulating inducible NO synthase (iNOS) (Cohen et al., 2000). In addition, P2X receptor family, long non-coding RNA (lncRNA), and inflammasome are also involved in MB-mediated anti-inflammation (Ahn et al., 2017; Li et al., 2018; Zheng and Li, 2019). Accordingly, MB application can be an important strategy to reduce inflammation and pain.

In general, voltage-gated sodium channels (VGSCs) play an important role in evoking action potentials (APs) and, when activated, they consequently contribute to exciting neurons and thus facilitating communication with other ones. Interestingly, MB decreased significantly I_{NA} (voltage-gated sodium currents) in hippocampal CA1 neurons and, more importantly, attenuated markedly neural firing rates in the afferent nerve fibers (Zhang et al., 2010; Lee et al., 2021), which implies that MB may impede pain transmission by dampening neuronal excitability elicited by VGSCs.

In addition, MB may contribute to pain reduction by hindering or damaging nerve connection to tissues, which is referred to as denervation. Indeed, it can make affected nerve fibers or neurons incapable of sensing pain. Peng et al. (2007) conducted intradiscal MB injection in patients with chronic discogenic low back pain (CDLBP) to relieve pain for the first time. Most of patients showed encouraging results and this improvement lasted at least one year. Moreover, in a case, there were no noticeable side effects and complications in those patients even after prolonged follow-ups (Peng et al., 2010). However, as opposed to expectations, these outstanding outcomes faced a lot of challenges. We will deal with the controversies around the results in the relevant section.

Despite these remarkable reports, MB has not drawn much attention from the public specifically concerning pain. Thus, in this review, we will show MB-driven analgesic effects and their possible mechanisms along with the relevant experimental evidence and clinical cases. Finally, we will provide a novel insight into MB as a pain reliever.

MB AND ANTI-INFLAMMATION

Blockade of NOS/NO-Mediated Inflammatory Signaling

Inflammation is tightly correlated with pain as a critical cause. It directly or indirectly induces nociceptive responses and in many cases underlies pain or pain-related diseases (Zhang H. et al., 2016; Harth and Nielson, 2019; Matsuda et al., 2019). It is well known that MB decreases NO formation by directly suppressing endothelial NOS (eNOS) expression, and blocks also the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) by suppressing soluble guanylate cyclase (sGC) expression in vascular smooth muscles, which in turn leads to vasoconstriction (**Figure 1A**; Wolin et al., 1990; Pan et al., 2019).

The MB-Induced Interruption of iNOS/NO-NF-κB Signaling

Based on this pathway, MB weakens inflammation since NO plays a crucial role in the pathogenesis of inflammation as a pro-inflammatory mediator. This dye inhibits iNOS/NO pathway to affect nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation. Silent information regulator 1 (Sirt1), which is a deacetylase and have a protective role under stress conditions, inactivates NF-κB and tumor protein p53 (p53) by deacetylating them. In contrast, iNOS/NO activates these two transcription factors by inactivating Sirt1 via S-nitrosylation (Nakazawa et al., 2017). NF-κB activation facilitates inflammatory cytokine expression, importantly, upregulates iNOS to enhance inflammatory signaling (Nakazawa et al., 2017; Jiang et al., 2020), and p53 activation induces cell death (Aubrey et al., 2018). However, MB prevents these events by downregulating iNOS in multiple inflammatory milieus. It directly inhibits iNOS expression and also attenuates the binding of NF-κB to iNOS promoter, thus resulting in the blockade of iNOS/NO-NF-κB signaling (**Figure 1B**; Huang et al., 2015).

Specifically, in human cartilage explants, MB decreased NO accumulation and iNOS expression in a dose dependent manner, and upregulated transforming growth factor beta (TGF-β) receptors, known as an important factor for cartilage matrix synthesis. As a result, it prevented the degradation of cartilage matrix and proteoglycan (Cohen et al., 2000). In ulcerative colitis rats, MB decreased remarkably NO production and inflammatory cytokine (IL-1β, IL-6, and TNF-α) levels. Functionally, with the alleviated tissue injury and edema in the submucosa, there was a significant improvement in intestinal permeability after MB application (Dinc et al., 2015). Accordingly, these studies demonstrated that MB has a critical role in negatively modulating NOS expression and NO production and ultimately is able to induce functional improvement in the inflamed tissues.

During nerve injury, Toll-like receptor 4 (TLR4) initiates neuroimmune activation in the nervous system. Notably, TLR4 activates NF-κB by mediating its nuclear localization, which in turn promotes pro-inflammatory cytokine expression and ultimately leads to inflammatory hyperalgesia (Lacagnina et al., 2018; Yadav and Surolia, 2019). Tanga et al. demonstrated for the first time that upon nerve injury (L5 nerve transection), behavioral hypersensitivity is induced via TLR4/NF-κB pathway, suggesting that TLR4 activation is a critical cause of the pathogenesis of neuropathic and chronic pain (Tanga et al., 2005). In this context, targeting TLR4/NF-κB pathway can be recommended as a decisive therapeutic strategy to relieve nerve injury–induced neuroinflammation and pain (Yadav and Surolia, 2019; Ye et al., 2020).

Interestingly, TLR4 is much involved in iNOS activation (Deng et al., 2015). This implies that MB may participate in suppressing the development and maintenance of neuropathic pain by downregulating directly iNOS and weakening NF-κB activation as previously described. A clinical study showed that systemic MB administration improved 10 patients with chronic refractory neuropathic pain, which was assumably due to the MB-mediated blockade of iNOS/NO pathway (Miclescu et al., 2015).

The MB-Induced Interruption of nNOS/cGMP/PKG Signaling

Lastly, neuronal NOS (nNOS), predominantly found in neurons, is also responsible for NO production and engaged in NO-mediated pathway (Kourosh-Arami et al., 2020). This enzyme has been much investigated specifically concerning chronic and inflammatory pain. In particular, nNOS has a critical role in the development of chronic and neuropathic pain upon nerve

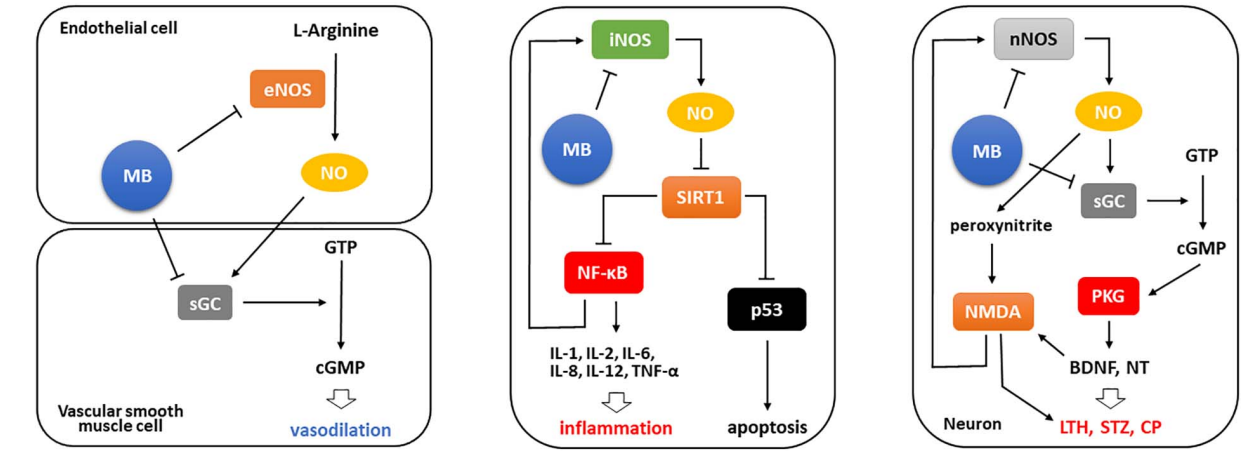

FIGURE 1 | MB is involved in anti-inflammation by suppressing iNOS/NO-NF-κB pathway. **(A)** Basically, MB downregulates both eNOS and sGC that are major factors converting GTP to cGMP, ultimately leading to vasoconstriction. **(B)** Upon tissue injury, iNOS functions as a strong inflammatory mediator in different types of cells. It inhibits Sirt1 activation by NO-mediated S-nitrosylation, which in turn activates NF-κB and p53 to facilitate inflammatory cytokine expression and apoptosis, respectively. Of note, NF-κB activation intensifies these events by activating iNOS/NO-NF-κB pathway. Conversely, MB directly abates iNOS expression and moreover decreases the binding of NF-κB to iNOS promoter, which consequently interrupts this inflammatory signaling. **(C)** Meanwhile, NMDA receptors are activated during nerve injury and induces Ca^{2+} influx, which then results in the excessive expression of nNOS and markedly activates nNOS/NO signaling. The increased NO production stimulates NMDA receptors and triggers NO/cGMP/PKG cascade, which promotes the subsequent BNDF upregulation and neurotransmitter release and ultimately induces long-term hyperexcitability and central sensitization. Notably, BDNF and peroxynitrite potentiate NMDA receptors, which stimulate nNOS expression again. However, MB weakens these responses by inhibiting nNOS and sGC activation, thus may prevent the development of persistent pain.LTH, long-term hyperexcitability; STZ, sensitization; NT, neurotransmitter; CP, chronic pain.

injury (Kim et al., 2011), while iNOS is partially related to it (Rocha et al., 2020). Interestingly, a study demonstrated that spinal nNOS, not eNOS and iNOS, is significantly upregulated after inflammatory pain induction and contributes to central sensitization (Chu et al., 2005). In this respect, nNOS has been highlighted as a target to dampen pain in both inflammatory and non-inflammatory milieus (Mukherjee et al., 2014; Demir et al., 2019). Moreover, it was found that NO/cGMP-mediated pathway, a downstream target of nNOS, is crucial for the development and maintenance of pain (Li et al., 2020), and cGMP protein kinase (PKG) is considered as a potent generator in this event since leading to long-term hyperexcitability and pain sensitization in neurons by inducing neurotransmitter release and upregulating brain-derived neurotrophic factor (BDNF) (Lewin and Walters, 1999; Sung et al., 2017; Wang et al., 2021). Furthermore, BDNF released during nerve injury and peroxynitrite activated via nNOS/NO signaling potentiate N-methyl-D-aspartate (NMDA) receptors, which then induces long-term hyperexcitability and upregulates nNOS, respectively (Pall, 2002; Chen et al., 2014; **Figure 1C**).

Importantly, MB is able to downregulate both nNOS and sGC, resulting in the inhibition of nNOS/NO-mediated signaling. Rey-Funes et al. (2016) demonstrated that MB protects retinal damage in rats with ischemic proliferative retinopathy, which is associated with local inflammation in the ischemic retina. MB suppressed the expression levels of nNOS and proangiogenic factors such as matrix metalloproteinase (MMP)-2 and MMP-9 and upregulated pigment epithelium-derived factor (PEDF), ultimately leading to the reduction of inner retinal thickness, gliosis, and retinal angiogenesis.

Inhibited NOS Activation Potentiates Opioidergic Effects

Opioids are substances with similar effects to those of morphine and used primarily for lessening pain. Basically, opioids act by binding to opioid receptors (ORs), which show analgesia by reducing cyclic adenosine monophosphate (cAMP) and causing the resultant decrease in the excitatory ion channels (Quirion et al., 2020; Sun et al., 2020; Kaski et al., 2021).

However, a number of issues concerning their addiction and adverse effects have been raised. In effect, morphine application may induce inflammatory cytokine expression such as IL-1β, IL-6, and TNF-α, ultimately contributing to inflammation and neurotoxic events. Importantly, it was found that reduced nNOS activation not only enhances the antinociception of morphine but also inhibits the morphine-induced neurotoxicity by downregulating inflammatory mediators (Machelska et al., 1997; Osmanlıoğlu et al., 2020). In this regard, MB may also contribute to these events as a potent NOS inhibitor. A clinical report showed that oral MB administration relieved oral mucositis-related intractable pain and more importantly reduced significantly morphine requirement in the relevant patients (Roldan et al., 2017).

There are some links between TLR4, OR, and cholecystokinin (CCK). CCK, an important neurohormone, plays a variety

of roles in the nervous system and notably engaged in pain reduction mainly via CCK-B receptors (Roca-Lapirot et al., 2019; Keppel Hesselink, 2020). Interestingly, this peptide functions to inhibit OR activity and thus reduces the morphine-induced antinociception (Torres-López et al., 2007; Yang et al., 2018). Meanwhile, upon nerve injury, *Tlr4* genes are significantly expressed in the midbrain and medulla of CCK-B receptor knockout mice compared to those of wild type, implying that CCK is involved in innate immunity (Kõks et al., 2008). This result is supported by recent studies that CCK has a pivotal role in anti-inflammation by decreasing inflammatory mediators (Funakoshi et al., 2019; Saia et al., 2020). It has not yet been confirmed that MB is related to these links. However, it is believed that MB possibly shows a synergistic effect with morphine and CCK, thus enhancing anti-inflammatory and analgesic effects.

Downregulation of P2XR and lncRNA

MB-Mediated P2XR Downregulation Induces Anti-inflammation and Pain Relief

Purinergic P2X receptor subtypes have been highlighted as a nociceptor that causes inflammation and pain. These receptors are characterized by ligand-gated ionotropic channels activated in response to the binding of adenosine 5′-triphosphate (ATP), exhibiting a non-selective cationic permeability such as K^+, Na^+, and Ca^{2+} that can contribute to opening voltage-gated channels on neurons by depolarizing membrane potential and activate a diversity of intracellular Ca^{2+}-dependent signaling pathways (Surprenant and North, 2009; Schmid and Evans, 2019). In particular, P2 × 3 and P2 × 2/3 receptors among the subunits are distributed in the dorsal root ganglion (DRG) and the central terminal of primary afferent fibers, mainly small-diameter unmyelinated C-fibers, and play a critical role in pain transmission in the periphery (North, 2004; Bernier et al., 2018; Stephan et al., 2018). Interestingly, MB is highly correlated with this P2X receptor-mediated events.

Li et al. showed that MB alleviated inflammation and pain in osteoarthritis (OA) rabbits by negatively modulating P2 × 3 receptors. In this study, after intra-articular MB administration, weight distribution in the hind paw was significantly restored and the swelling ratio in the inflamed knees also markedly declined. Inflammatory cytokine levels (TNF-α, IL-1β, IL-6, and IL-8) were also remarkably reduced in the articular cartilage with a significant decrease in P2 × 3 receptor expression. Moreover, this event was reversed by P2 × 3 overexpression (Li et al., 2018).

Decreased lncRNA Contributes to Anti-inflammation

Meanwhile, lncRNA has been highlighted as a new player in gene regulation. Previous studies revealed that increased lncRNA is a critical cause of neuropathic pain, and interacts with P2XRs to initiate and maintain pain (Zhao et al., 2013; Li et al., 2017). Also, it is much implicated in inflammatory events (Chen et al., 2019).

Interestingly, MB may contribute to anti-inflammation and pain relief by downregulating this lncRNA. Zheng and Li demonstrated that MB negatively regulates a long non-coding RNA (lncRNA), specifically a chondrocyte inflammation–associated lncRNA (CILinc02), overexpressed in osteoarthritic cartilage tissues and cultured OA cells, and decreased inflammatory cytokine levels (IL-1, IL-6, and IL-17). Of note, MB suppressed even chondrocyte degradation. Conversely, CILinc02 overexpression resulted in inflammation and apoptosis (Zheng and Li, 2019).

Inhibition of Inflammasome Formation and Activation

Inflammasome is an intracellular multiprotein complex that functions to process cytokine precursors into their mature forms and induce an inflammatory form of programmed cell death termed pyroptosis, which, ultimately, has a crucial role in providing host defense against harmful intruders (Matsuda et al., 2019).

Upon stimulation, inflammasome initiates the expression of cytosolic sensing proteins, called a priming step, which include nucleotide-binding oligomerization domain (NOD), leucine-rich repeat (LRR), pyrin domain-containing protein 3 (NLRP3), NLR family caspase recruitment domain (CARD)-containing protein 4 (NLRC4), absent in melanoma 2 (AIM2), and cytokine precursors. And inflammasome requires the second phase triggered by pathogen- and danger-associated molecular patterns, an activation step, to assemble a cytosolic sensor, an adaptor [apoptosis-associated speck-like protein containing a CARD (APC)], and an effector (pro-caspase-1) into inflammasome complex, and to secrete IL-1β and IL-18 (Kanneganti, 2015; Rathinam and Fitzgerald, 2016; Swanson et al., 2019; Yang et al., 2019).

Interestingly, it was found that MB inhibits inflammasome activation by interrupting both steps. Ahn et al. elucidated the relation between MB and inflammasome for the first time, demonstrating that MB suppresses both canonical and non-canonical processes by decreasing dose-dependently the expression of NLRP3, pro-IL-1β and caspase-1, as well as reactive oxygen species (ROS) production (Ahn et al., 2017).

Similarly, neuroinflammation triggered by spinal cord injury (SCI) was alleviated by the MB-mediated inhibition of NLRP3 and NLRC4 inflammasome formation in microglia. In SCI animals, following MB administration, their locomotive activities were ameliorated and inflammatory cytokine levels (IL-1β, IL-6, and TNF-α) were also diminished (Lin et al., 2017). Overall MB-mediated anti-inflammation pathways are summarized in **Table 1**.

MB AND SODIUM CURRENT REDUCTION

Early Studies for MB and I_{NA}

It is an old story that MB is involved in the reduction of excitatory synaptic currents. In earlier periods, Armstrong, Croop, and Starkus conducted pioneering experiments to explore the inactivation of sodium channels and their electrophysiological profiles using an artificial inactivation model established by administering MB into squid giant axons. The dyes blocked efficiently both the I_g (gate current) and I_{NA}, as well as

simulated the channel inactivation even after pronase, an agent of eliminating normal inactivation, treatment (Armstrong and Croop, 1982). Similarly, a subsequent study showed that MB weakened sodium currents in both normal and pronase-treated crayfish giant axons (Starkus et al., 1984). In the two studies, MB was believed to be a gate-immobilizing open-pore blocker rather than an inactivation enhancer, since directly blocking sodium pores rather than promoting or speeding the inactivation process (**Figure 2**; Armstrong and Croop, 1982; Starkus et al., 1984).

Altered VGSC-Dependent Currents and Neural Firing Rates

Zhang et al. (2010) explored the effect of MB on sodium currents and found that MB alters VGSC-mediated electrophysiological events. MB application diminished the peak amplitude of I_{NA} up to 45% at 100 μM, number of repetitive firings, and V_{max} (AP upstroke velocity) in rat hippocampal slices. In addition, it accelerated I_{NA} inactivation and even retarded the shift from inactivation to recovery state.

Importantly, Lee et al. (2021) performed the first *in vivo* experiments to investigate the link between MB-mediated neural firing patterns and pain reduction in rats using *in vivo* single nerve recordings and behavioral studies. This study showed dramatic results that neural firing rates significantly decreased in a dose-dependent manner after MB administration and, notably, this event lasted longer than that of lidocaine, showing anesthetic-like firing patterns. Ultimately, MB improved pain behaviors in rodents. These results demonstrate that MB abates pain by markedly suppressing neural excitability (Lee et al., 2021).

Silenced Excitable Neurons as a Critical Cause of Pain Relief

Exciting neurons is the most fundamental step to convey signals and to relay or cause several subsequent responses. Moreover, VGSCs are directly engaged in AP generation and affect the

TABLE 1 | MB-mediated multiple anti-inflammation pathways.

Pathways	Changes in key elements	Final results	References
MB-NOS/NO	iNOS expression ↓ NO production ↓ TGF-β receptor expression ↑	cartilage matrix and proteoglycan degradation ↓ (in cultured human cartilage explants)	Cohen et al., 2000
	iNOS expression ↓ NO production ↓ IL-1β, IL-6, TNF-α expression ↓	tissue injury and edema ↓ (in rats with acetic acid-induced colitis)	Dinc et al., 2015
	iNOS expression ↓ NO production ↓	NF-κB binding to iNOS promoter ↓ (in mouse organs and cultured cells)	Huang et al., 2015
	nNOS expression ↓ MMP-2, MMP-3 expression ↓ PEDF expression ↑	inner retinal thickness ↓ gliosis ↓ retinal angiogenesis ↓ (in retinopathy rats)	Rey-Funes et al., 2016
MB-P2 × 3	P2 × 3R expression ↓ IL-1β, IL-6, IL-8, TNF-α expression ↓	swelling ratio in the inflamed knee ↓ weight distribution in hind paws ↑ (in OA rabbits)	Li et al., 2018
MB-lncRNA	CILinc02 expression ↓ IL-1, IL-6, and IL-17 expression ↓	chondrocyte degradation ↓ inflammation ↓ (in human cartilage tissues and primary cells)	Zheng and Li, 2019
MB-inflammasome	NLRP3, pro-IL-1β expression ↓ IL-1β, IL-18, caspase-1 expression ↓ mitochondrial ROS production ↓	inflammasome activation ↓ (in bone marrow-derived macrophages)	Ahn et al., 2017
	NLRP3 expression ↓ ROS production ↓ IL-1β, IL-6, IL-18, TNF-α expression ↓	NLRP3 and NLRC4 inflammasome formation ↓ neuroinflammation ↓ locomotive function ↑ (in cultured microglia and spinal cord injured rats)	Lin et al., 2017

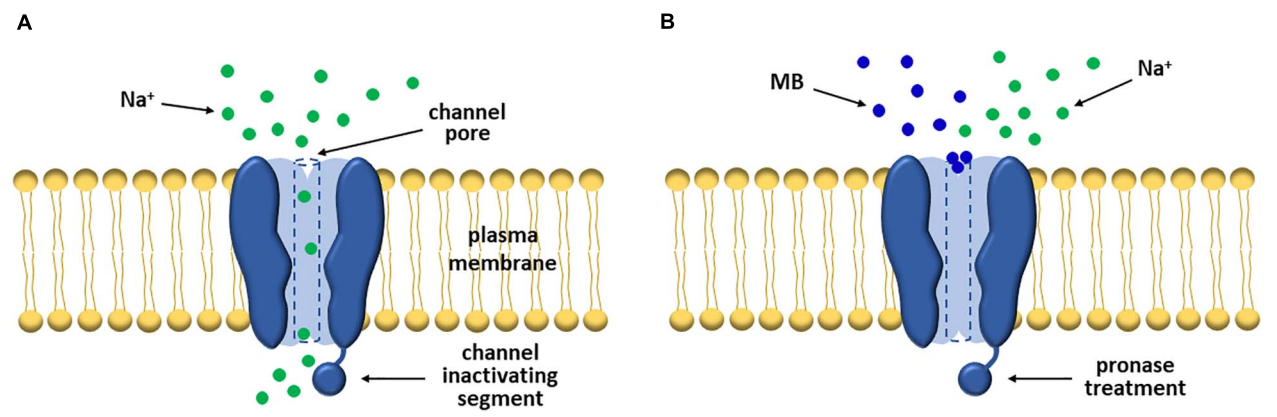

FIGURE 2 | MB significantly attenuates sodium currents by blocking VGSCs. **(A)** In general, VGSCs allow sodium ions to flow into the cell in the activated state. **(B)** However, early researchers found that the gate and sodium currents of the channels were markedly suppressed post-MB treatment. And notably, this event was maintained even after pronase treatment. Thus, they interpreted this event as MB functions as a pore blocker rather than an inactivation enhancer.

resultant reactions including signal propagation, opening other channels, vesicle release, cell-to-cell communication, and other related responses (Kruger and Isom, 2016; Wang et al., 2017). Accordingly, it is natural to postulate that VGSCs and AP generation play a crucial role even in pain transmission (Dubin and Patapoutian, 2010; Bennett et al., 2019). Interestingly, multiple evidence demonstrates that MB silences excitable cells (or nerves) by significantly attenuating I_{NA} and AP production, which ultimately leads to pain relief (Armstrong and Croop, 1982; Starkus et al., 1984, 1993; Zhang et al., 2010; Lee et al., 2021). Therefore, these two crucial electrophysiological activities, weakened sodium currents and decreased neural firing rates, are possible causes that can elucidate how MB contributes to pain relief.

MB AND DENERVATION

Early Cases for MB Application

Denervation is defined as loss or interruption of nerve connection to an organ, which thereby may contribute to pain relief by making nerves shut off sensory transmission. In history, MB began to rear its head for denervation firstly to treat itch. In 1968, Professor Rygick in the Moscow University reported for the first time an effect of MB on chronic pruritus ani (PA), characterized by severe itching in the perianal area, noting that patients with chronic PA was significantly improved after subcutaneous MB administration (Rygick, 1968). In 1979, Wollock and Dintsman inspired by Rygick's work conducted also an intervention to treat intractable PA locally administering MB into the perianal skin and produced remarkable results that eight out of nine patients were completely improved post-MB treatment. Importantly, Eusebio et al. conducted an electron microscopic investigation aimed at the perianal skin of intractable PA patients treated with subcutaneous MB and no distinct nerve endings were detected in the samples even after a long-term (7 years) follow-up. Thus, they interpreted that this improvement was attributed to death of nerve endings connected to the perianal skin, that is, denervation (Eusebio et al., 1990). Most recently, PA patients treated with MB showed satisfactory scores at 6-week, as well as 3-year, follow-ups. The recurrence rate was low (7.5%) even three years after MB treatment. In the study, MB was also considered as a critical agent to sever nerve endings (Kim et al., 2019).

Intradiscal MB Injection and Debates

The Novel Intervention to Treat Chronic Pain

Similar to the itching cases, MB was applied to inflamed intervertebral disks (IVDs) in CDLBP patients. In 2007, Peng et al. (2007) reported encouraging outcomes in the treatment of CDLBP using a minimal invasive method, intradiscal MB injection. They believed that MB has a capacity to mitigate pain in the patients by blocking nerve conduction or destroying nerve endings around the painful disks that may be vascularized and extensively innervated owing to disk degeneration. This was a first prospective clinical trial conducted for examining the pain-relieving effect of MB in human subjects. In this study, MB was administered into the affected disks of CDLBP patients using a discographic needle (1 ml of 1% MB), then pain intensity and disability of the patients were measured by visual analog scale (VAS) and oswestry disability index (ODI). As a result, most patients were evidently or completely improved and there were no side effects or complications during long-term follow-up periods (12-23 months) (Peng et al., 2007). After four years, Peng et al. (2010) conducted a randomized placebo-controlled trial in 72 eligible participants, who accepted intradiscal MB injection or placebo treatment. For numerical rating scale (NRS) and ODI scores, there were dramatic or obvious improvements in most patients treated with MB. Of note, these events lasted even to the subsequent follow-up visits at 12 and 25 months.

Controversies and Refutations for Intradiscal MB Application

However, their outcomes were immediately challenged and debated over a more fundamental issue. Bogduk (2010) explained that cultural factors may affect the manner in which patients report their outcomes, and Chinese patients may report their physical conditions more favorably to physicians or assessors than United States or British patients do. Another commentary was about the safety of MB. MB has a potent neurotoxic effect and can be leaked to the spinal canal through annular tears during intradiscal administration since discogenic pain is associated with annular tears (Levine and Richeimer, 2011). Lastly, a researcher argued that there is no LBP treatment that can immunize against new LBP episodes, emphasizing that discography accelerates disk degeneration after years (Schiltenwolf et al., 2011).

In 2019, an article directly refuted the previous results of Peng et al. (2010) designing a study protocol almost identical to their previous study to verify their outcomes (Kallewaard et al., 2019). As a result, there were no significant differences between MB plus lidocaine treatment group and placebo plus lidocaine group after NRS, ODI, quality of life (QOL), and VAS measurement. They concluded that the outcomes of the previous study were not able to be reproduced (Kallewaard et al., 2019).

Short-Term Effect of Intradiscal MB Application on Chronic Low Back Pain

Over years after Peng et al. (2010), there have been multiple attempts to perform intradiscal MB injection in CDLBP patients. Kim et al. revealed that following intradiscal MB injection, there was a significant decrease in VAS (1, 3, and 6 months) and ODI (one and three months) scores in 20 CDLBP patients, but, one year after intervention, such outcomes were reproduced only in five patients (Kim et al., 2012). Accordingly, the patients showed short-term improvement (three or six months) after intervention. Gupta et al. showed a relatively low success rate (13%) in eight CDLBP patients after a single intradiscal MB injection (Gupta et al., 2012). Levi et al. revealed that there were very limited benefits in the VAS and ODI scores in 16 CDLBP patients. Only a few patients (25% or less) met the criteria for success at the follow-up periods (Levi et al., 2014). Lastly, Zhang X. et al. (2016) reported the short-term clinical outcomes in CDLBP patients. Their NRS and ODI scores were significantly decreased at 1- to 6-month follow-ups. Imaging experiments showed that the mean

apparent diffusion coefficient (ADC) and T2 values significantly increased at 6- and 12-month, but not 3-month, follow-ups.

Collectively, it was found that MB is effective in CDLBP patients at least in a short period (until 3 or 6 months after treatment), which is also supported by the most recent review report (Deng et al., 2021). Similarly, radiofrequency (RF) denervation also resulted in short-term improvement (4 weeks or 6 months) in patients with chronic LBP (Leclaire et al., 2001; Nath et al., 2008; Al-Najjim et al., 2018). In addition, epidermal nerve fibers were regenerated in healthy humans about 100 days after capsaicin-induced denervation (Polydefkis et al., 2004). Regarding knee osteoarthritis, RF denervation contributed to pain reduction in patients with inflamed knee joints and their joint function was ameliorated. This improvement lasted 6 and 3 months, respectively (Wang R. et al., 2019). Based on these results, it is believed that denervating effects do not exceed 6 months. Accordingly, the short-term improvement observed in CDLBP patients is interpreted reasonable. Overall results of the studies were summarized in **Table 2**.

MB AND TOXICITY ISSUES

Serotonin Toxicity

The Contribution of MB to Serotonin Toxicity as a Potent MAO Inhibitor

In the last 20 years, it has been reported that depressive or anxiety patients taking serotonergic medications experienced ST, also referred to as serotonin syndrome, after MB administration. The investigators and medical doctors have believed that the toxicity is deeply linked with the synergism of MB and the medications, and that such patients may be more vulnerable to ST (Ng et al., 2008; Ng and Cameron, 2010; Kapadia et al., 2016; Wolvetang et al., 2016).

ST is characterized by the excessive accumulation of serotonin into the body, which, ultimately, leads to neuromuscular hyperexcitability by the excessive serotonergic agonism of the central and peripheral nervous system, whose clinical findings include agitation, tremor, inducible and ocular clonus, diaphoresis, hyperreflexia, hypertonia, and hyperthermia (over 38°C) (Boyer and Shannon, 2005). A great number of case reports showed that these events occurred predominantly in mental patients who had been taking serotonergic antidepressant medications including fluoxetine [a selective serotonin reuptake inhibitor (SSRI)] (Martindale and Stedeford, 2003; Kapadia et al., 2016), paroxetine, a SSRI (Bach et al., 2004; Mihai et al., 2007; Ng et al., 2008; Shanmugam et al., 2008; Schwiebert et al., 2009; Wolvetang et al., 2016), venlafaxine [a selective serotonin and norepinephrine reuptake inhibitor (SSNRI)] (Majithia and Stearns, 2006), citalopram, a SSRI (Mathew et al., 2006; Pollack et al., 2009), duloxetine, a SSNRI (Rowley et al., 2009), and clomipramine [a serotonin reuptake inhibitor (SRI)] (Khan et al., 2007), and have been commonly interpreted to be attributed to the reaction of MB as a potent monoamine oxidase A (MAO-A) inhibitor (Gillman, 2011; Top et al., 2014). In effect, MAO has a part in the degradation process of a diversity of monoamines such as serotonin, epinephrine, norepinephrine, dopamine, and histamine (Yeung et al., 2019; Floris et al., 2020). In this regard, MB augments serotonin levels in the synaptic cleft by suppressing the activity of MAO, which, furthermore, may lead to ST along with the use of SRIs, SSRIs, and SSNRIs due to over-enhanced serotonergic transmission (Zuschlag et al., 2018).

The Risk of Intravenous MB Application in Patients Taking Serotonergic Medications

Previous studies showed that ST was precipitated after intravenous MB administration predominantly at doses of 5-7.5 mg/kg except for the case of a 65-year-old woman (1.75 mg/kg) (Mihai et al., 2007; Ng et al., 2008). More importantly, Gillman reported that even a low dose of MB (0.75 mg/kg) administered via the intravenous route may reach peak plasma concentration and cause CNS toxicity (or ST) in those being treated with the medications facilitating serotonergic transmission (Schwiebert et al., 2009; Gillman, 2011). The drug safety update, a newsletter issued by the Medicines and Healthcare products Regulatory Agency (MHRA), also informed that patients who have recently treated with serotonergic antidepressants should avoid intravenous MB and be closely observed if administered with MB, and that intravenous MB can be approved only for patients with drug-induced methemoglobinemia at a dose of 1–2 mg/kg (MHRA, 2009). Oral administration of MB is not likely to cause ST since the MB concentration in blood and brain was significantly higher after intravenous, compared to oral, administration (Peter et al., 2000; Top et al., 2014). Therefore, we need to avoid intravenous route and consider about proper dosage to prevent this toxicity event.

TABLE 2 | Overall results of intradiscal MB Injection in CDLBP Patients.

Case report	Number of patients	Measurements	Duration of pain relief	Success rate
Peng et al., 2007	24	VAS, ODI	23 mo	87% at 3, 6, and 12 (or more) mo
Peng et al., 2010	36 (MB) 36 (placebo)	NRS, ODI	24 mo	89% at 6, 12, and 24 mo
Kim et al., 2012	20	VAS, ODI	12 mo	55% and 20% at 3 and 12 mo
Gupta et al., 2012	8	retrospective analysis	12 mo	13% at 12 mo
Levi et al., 2014	16	VAS, ODI	6 mo	25%, 21%, and 25% at 1, 2, and 6 mo, respectively
Zhang X. et al., 2016	33	NRS, ODI	12 mo	81, 75, 63 and 54% at 1, 3, 6, and 12 mo, respectively
Kallewaard et al., 2019	40 (MB) 41 (placebo)	NRS, PGIC, VAS, ODI, QoL	6 mo	15(12)%, 25(20)%, and 35(25)% at 6 wk, 3 mo, and 6 mo in NRS (PGIC), respectively

PGIC, patent global impression of change; wk, week(s); mo, month(s).

Other Issues

A previous study demonstrated that MB deteriorated the dendritic arbor of isolated neurons and was engaged in cell death in a dose-dependent manner (no neurotoxic effects at concentrations of 0.25 μM or less) (Vutskits et al., 2008). More recently, it was found that MB has a detrimental effect on the viability of nucleus pulposus and annulus fibrosus cells. But MB at lower doses had little effect on both (Wang X. et al., 2019; Zhang et al., 2019). Thus, these results imply that high-dose MB may impair them, thus we need to consider about proper dosage in the clinical setting.

DISCUSSION

In the present review, we illustrated MB-driven analgesic effects and their possible mechanisms based on key clinical and experimental studies. In effect, MB is much closely involved in pain relief via the following major actions: anti-inflammation, sodium current reduction, and denervation (**Figure 3**).

First of all, MB is deeply related to anti-inflammation. Notably, it triggers a variety of anti-inflammatory pathways and functions to decrease the expression level of pro-inflammatory cytokines in a variety of ways, including inhibition of iNOS/NO signaling, downregulation of P2XR and lncRNA, and interruption of priming and activation steps required for inflammasome complex formation and activation. Ultimately, these anti-inflammatory responses contribute to restoring inflamed tissues and lessening pain (**Table 1**; Vutskits et al., 2008; Dinc et al., 2015; Ahn et al., 2017; Li et al., 2018; Zheng and Li, 2019). In addition, MB reduces the amplitude of I_{NA}, accelerates the I_{NA} inactivation, delays the shift from inactivation to recovery state in neurons, and importantly decreases neural firing rates, which can therefore alleviate pain by impeding VGSC-mediated neuronal excitability (Zhang et al., 2010; Lee et al., 2021). Lastly, MB is involved in denervation. MB has been believed to have a neurolytic effect and thus to destroy dermal nerve endings for years. Of note, this effect was confirmed by an electron microscopic experiment (Eusebio et al., 1990; Etter and Myers, 2002). In clinical cases, CDLBP patients were improved for at least 3 or 6 months after intradiscal MB injection and RF denervation (Kim et al., 2012; Maas et al., 2015; Zhang X. et al., 2016; Al-Najjim et al., 2018).

However, there are toxicity problems with MB application. Patients being treated with serotonergic medications are likely to suffer ST after intravenous MB administration (mainly at doses of 5-7.5 mg/kg) due to the synergistic effect of MB and the medications. In addition, MB is able to damage cultured neurons (Vutskits et al., 2008) and NP and AF cells in a dose-dependent manner (Wang X. et al., 2019; Zhang et al., 2019). Therefore, we should find proper dose levels and administration routes to protect MB-induced adverse events.

MB is a blue dye that had been used at first for textile manufacturing. Similarly, at the present day, it is applied as a dye to mark and visualize a certain tissue or region in the

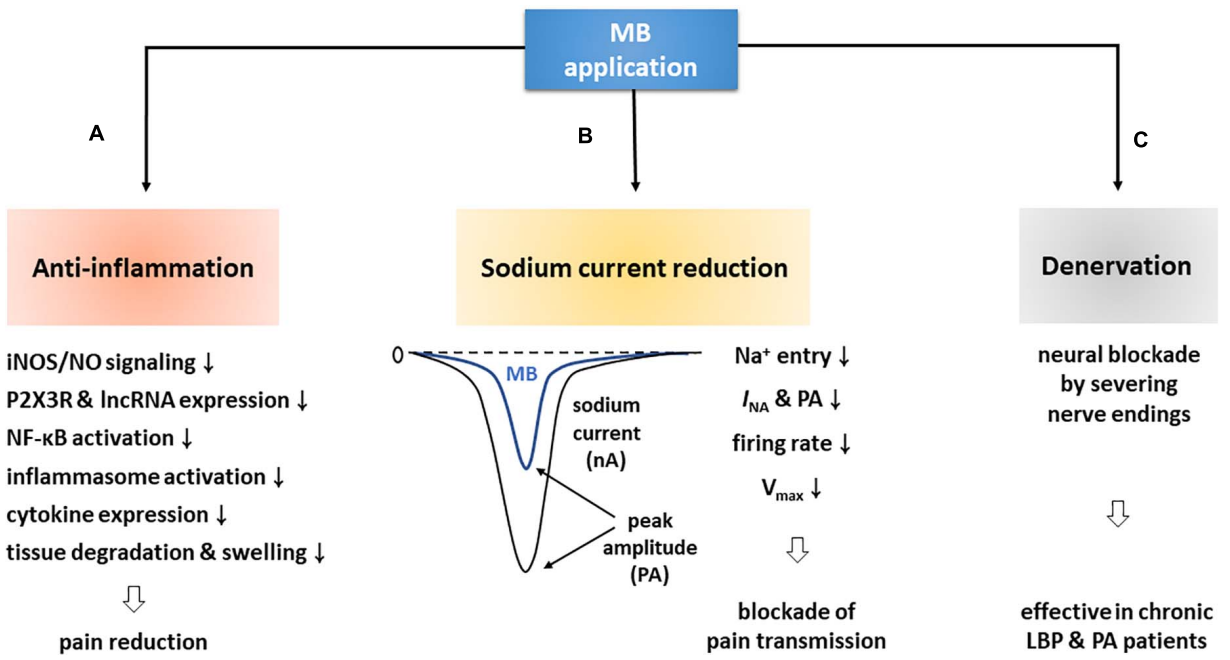

FIGURE 3 | MB contributes to pain reduction via three major routes. **(A)** First of all, MB is deeply involved in anti-inflammation. MB application blocks iNOS/NO signaling by downregulating iNOS, and suppresses P2 × 3R and lncRNA expression, NF-κB activation, and inflammasome formation, which thereby decreases inflammatory cytokine levels. These events are ultimately followed by pain reduction with the prevention of tissue degradation and swelling. **(B)** In addition, MB application attenuates neuronal excitability by decreasing I_{NA} and firing rates. These altered electrophysiological properties may contribute to pain relief by blocking synaptic transmission. **(C)** Lastly, MB application improved chronic PA and LBP. An electron microscopic experiment demonstrated that such efficacy was due to the death of nerve endings.

clinical and experimental settings. However, astonishingly, MB has been also used in biological and medical studies for a long time. Importantly, this review highlighted the relation between MB and pain reduction and made an effort to inform the relevant mechanisms. MB is engaged in pain reduction via diverse routes, but there is a still lack of scientific evidence. There are still a lot of blanks to be filled in the anti-inflammatory pathways and remain possible links to be uncovered between MB and pain-related receptors.

Modern medicine lies in the positivist tradition, which implies that medicine must be based on a solid foundation and the foundation can be constructed by the faithful observation of life phenomena (Cabanis, 1803). In addition, the certainty of medical facts can be guaranteed by that of experimental science such as experimental certainty and logical certainty. The former can be obtained by senses of observant subjects, the latter by intellectual action of humans (Bouillaud, 1836). With regard to the relation between MB and pain control, a solid foundation has not been established yet, although MB intercalates into critical signal pathways to reduce inflammation and pain. Thus, we need still further investigations to build the foundation and reach the certainty of medical facts.

Lessening pain is one of the salient issues in the medical profession, which may also determine our QoL. A great number of medications have been explored and are now applied to patients to relieve pain. Given the actions of MB in pathological or inflammatory milieus, this review offers a strong possibility that this dye may be developed as a new therapeutic agent for pain patients.

AUTHOR CONTRIBUTIONS

First of all, HH and SL set up the concept of this study, were involved in its overall design, and wrote this manuscript. Moreover, SL put a lot of effort into acquiring and analyzing the relevant articles and data, and made tables and figures to illustrate more clearly methylene blue-mediated events and mechanisms. Both authors contributed to completing this manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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