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INVITED REVIEW

Minocycline targets multiple secondary injury mechanisms in traumatic spinal cord injury

Robert B. Shultz, Yinghui Zhong*
School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA

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Abstract

Minocycline hydrochloride (MH), a semi-synthetic tetracycline derivative, is a clinically available antibiotic and anti-inflammatory drug that also exhibits potent neuroprotective activities. It has been shown to target multiple secondary injury mechanisms in spinal cord injury, *via* its anti-inflammatory, anti-oxidant, and anti-apoptotic properties. The secondary injury mechanisms that MH can potentially target include inflammation, free radicals and oxidative stress, glutamate excitotoxicity, calcium influx, mitochondrial dysfunction, ischemia, hemorrhage, and edema. This review discusses the potential mechanisms of the multifaceted actions of MH. Its anti-inflammatory and neuroprotective effects are partially achieved through conserved mechanisms such as modulation of p38 mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt signaling pathways as well as inhibition of matrix metalloproteinases (MMPs). Additionally, MH can directly inhibit calcium influx through the N-methyl-D-aspartate (NMDA) receptors, mitochondrial calcium uptake, poly(ADP-ribose) polymerase-1 (PARP-1) enzymatic activity, and iron toxicity. It can also directly scavenge free radicals. Because it can target many secondary injury mechanisms, MH treatment holds great promise for reducing tissue damage and promoting functional recovery following spinal cord injury.

Key Words: *minocycline; inflammation; anti-oxidant; neuroprotection; oxidative stress; glutamate exitotoxicity; cytochrome c; P38 MAPK; PI3K/Akt; calcium influx*

Correspondence to:
Yinghui Zhong, Ph.D.,
yz348@drexel.edu.

orcid:
0000-0002-1363-9466
(Yinghui Zhong)

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Introduction

Traumatic spinal cord injury (SCI) causes deleterious functional loss below the level of injury. The initial trauma results in rapid hemorrhage and cell death, and offers little opportunity for therapeutic intervention. Following the initial trauma, secondary injury cascades occur, causing widespread and persistent inflammation and progressive tissue loss. During this stage, lesions can become many times larger than the initial injury (Fitch et al., 1999; Rossignol et al., 2007; Fehlings and Nguyen, 2010). Therapies that can inhibit secondary injury progression thus offer a promising and clinically viable approach to reduce tissue damage and functional deficits following SCI.

Many mechanisms contribute to the secondary injury, including inflammation, cellular damage from free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), glutamate exitotoxicity, calcium influx, ischemia, hemorrhage, and edema (Oyinbo, 2011). However, most current therapies only target one or a few elements of the secondary injury mechanisms, and have been largely unsuccessful in clinical trials (Thuret et al., 2006; Lammertse, 2013; Varma et al., 2013).

Minocycline hydrochloride (MH), a semi-synthetic tetracycline derivative, is a clinically available antibiotic and anti-inflammatory drug that also exhibits potent neuroprotective activities. It can potentially target a broad range of secondary injury mechanisms, and protect neural tissue from multiple neurotoxic insults after SCI, *via* its anti-inflammatory, anti-oxidant, and anti-apoptotic properties (Stirling et al., 2005; Elewa et al., 2006; Sapadin and Fleischmajer, 2006; Plane et al., 2010; Ghazali et al., 2016; Chin et al., 2017). MH has been shown to (1) inhibit inflammatory processes contributing to progression of secondary injury (Lee et al., 2003a); (2) protect neurons from oxidative stress and scavenge free radicals (Lee et al., 2003a); (3) inhibit inducible nitric oxide synthase (iNOS) that produces nitric oxide (NO) (Amin et al., 1996); (4) prevent glutamate-induced apoptosis of neurons (Pi et al., 2004); (5) prevent N-methyl-D-aspartate (NMDA)-induced excitotoxicity by diminishing NMDA-induced Ca^{2+} influx and mitochondria Ca^{2+} uptake (Garcia-Martinez et al., 2010); (6) prevent apoptosis by inhibiting mitochondrial cytochrome c (CytC) release after SCI (Teng et al., 2004); (7) inhibit oligodendrocyte apoptosis and improve functional recovery after SCI (Stirling et al., 2004); (8) protect grey and white matter from spinal cord ischemia (Takeda et al., 2011); (9) protect neurons from hemorrhage-induced toxicity (Takeda et al., 2011); and (10) protect blood-brain barrier and reduces edema following intracerebral hemorrhage (Wasserman and Schlichter, 2007). Thus, MH can serve as a multifaceted agent that targets multiple mechanisms contributing to secondary injury and has great therapeutic

potential for the treatment of SCI.

Although there is a wealth of evidence supporting the efficacy of MH treatment following SCI in animal models, a comprehensive discussion of the multiple mechanisms of action within this context is missing. The mechanisms of action can be classified into three categories: (1) anti-inflammatory activity; (2) anti-oxidative activity; and (3) direct neuroprotective activity. In this review, we discuss the possible mechanisms by which MH exerts these effects to reduce secondary injury after SCI.

Mechanisms of Anti-Inflammatory Activity

Inflammation is a key mediator of secondary injury progression in SCI. Following initial injury, resident microglia become activated to pro-inflammatory phenotypes, while blood-borne factors and leukocytes infiltrate the spinal cord tissue (Byrnes et al., 2006; Zhou et al., 2014). In the mileu of cellular signals that follow, a complex network of cross-talk is established among recruited peripheral leukocytes, resident microglia, and astrocytes, resulting in further upregulation of neurotoxic and pro-inflammatory cytokines and chemokines (McTigue et al., 1998; Gonzalez et al., 2003; Pineau and Lacroix, 2007; Stammers et al., 2012); increased production of cytotoxic ROS/RNS (Xu et al., 2005; Cooney et al., 2014); upregulation of regeneration-inhibitory molecules including proteoglycans and the myelin-derived inhibitors Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) (Filbin, 2003; Schweigreiter and Bandtlow, 2006; Yiu and He, 2006; Dou et al., 2009); and formation of the inhibitory glial scar (Pekny and Nilsson, 2005; Yiu and He, 2006). While inflammation has also been shown to promote clearance of debris and regeneration following SCI (David et al., 2012), therapeutic strategies that mitigate inflammation have been shown to promote cell survival and functional recovery after SCI (Lee et al., 2003a; Stirling et al., 2004; Wang et al., 2017), probably because inflammation is excessive at least at the acute stage (Gensel and Zhang, 2015). MH has been found to modulate inflammation through a number of pathways—a detailed illustration is presented in **Figure 1**.

Regulation of pP38 mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt inflammatory signaling pathways

Inflammation is associated with activation (phosphorylation) of p38 MAPK (**Figure 1**), a protein kinase with a number of pro-inflammatory downstream effects (Yang et al., 2014). Activation of p38 MAPK results in activation and/or nuclear translocation of pro-inflammatory transcription factors, including nuclear factor kappaB (NF-κB) (Olson et al., 2007), lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) (Ceccarelli et al., 2015), Nur77 (Pang et al., 2012), activator protein 1 (AP-1) (Slomiany and Slomiany, 2013), and activating transcription factor 2 (ATF-2) (Hirose et al., 2009). These transcription factors regulate synthesis of leukocyte-recruiting chemokines and pro-inflammatory cytokines, including monocyte-chemoattractant protein-1 (MCP-1) (Hacke et al., 2010), tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Olson et al., 2007; Pang et al., 2012; Yu et al., 2014). MH has been shown to inhibit phosphorylation of p38 MAPK (Yune et al., 2007; Corsaro et al., 2009; Pang et al., 2012), reduce activation and translocation of inflammation-associated transcription factors such as NF-κB, LITAF, and Nur77 (Pang et al., 2012; Song et al., 2016), and decrease expression of pro-inflammatory cytokines and chemokines both *in vitro* and *in vivo* (Lee et al., 2003b; Kielian et al., 2007; Cai et al., 2010; Pang et al., 2012; Switzer et al., 2012). MH treatment has also been shown to maintain activation of PI3K/Akt (Pang et al., 2012; Hahn et al., 2016), a negative regulator of p38 MAPK (Guha and Mackman, 2002). Inhibition of PI3K/Akt and subsequent stimulation of p38 MAPK was shown to ameliorate the effects of MH on transcription factor activation/translocation and cytokine expression (Pang et al., 2012), suggesting that MH regulation of transcription factors and cytokine/chemokine expression are, at least in part, downstream effects of p38MAPK inhibition.

Inflammation-induced p38MAPK activation also leads to increased expression of iNOS (Choi et al., 2005; Sung et al., 2012) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit (Spencer et al., 2016). NO and superoxides produced by iNOS and NADPH oxidase are highly reactive species capable of damaging cell membranes, proteins, nucleic acids, and organelles, resulting in cell death. At the same time, NO also acts as a pro-inflammatory signaling molecule (Korhonen et al., 2005; Sharma et al., 2007), while superoxides can participate in the formation of lipid-derived pro-inflammatory signaling molecules (Yadav and Ramana, 2013). MH has been shown to reduce iNOS expression and NO production from reactive microglia and macrophages (Amin et al., 1996; Zhang et al., 2014), and to prevent the overexpression of NADPH oxidase in the remission phase of experimental multiple sclerosis (MS) microglia (Di Filippo et al., 2016). No direct effect of MH was found on the enzymatic activity of iNOS, suggesting a regulatory effect on gene expression was responsible for decreases in NO production (Amin et al., 1996). It is possible that MH regulation of iNOS/NADPH oxidase expression is a downstream effect of inhibiting p38 MAPK pathway.

Inflammation-activated p38 MAPK has also been shown to regulate microglial expression of proNGF, a nerve growth factor (NGF) precursor (Yune et al., 2007). ProNGF has been shown to act as a distinct ligand, activating a death-inducing receptor complex in neurons (Nykjaer et al., 2004; Hempstead, 2009) and inducing oligodendrocyte death following SCI (Beattie et al., 2002). MH has been shown to inhibit p38 MAPK activation and microglial proNGF expression following SCI, resulting in improved oligodendrocyte survival (Yune et al., 2007). In addition, treatment with a specific p38 MAPK inhibitor reduced proNGF expression from lipopolysaccharide (LPS) stimulated microglia *in vitro*. This data clearly illustrates that p38 MAPK activation is a prerequisite

for inflammation-induced proNGF expression that can be targeted by MH treatment.

Regulation of phospholipase A2

Inflammation also results in upregulation and activation of a class of enzymes known as phospholipase A2s (PLA2s) that break down membrane phospholipids, yielding arachidonic acid (AA). AA is then metabolized into prostaglandins and leukotrienes by cyclooxygenase (COX) and lipoxygenase (LOX), respectively. Both prostaglandins and leukotrienes are potent pro-inflammatory mediators that are then secreted into the extracellular space (Balsinde et al., 2002). Prostaglandins and leukotrienes have been shown to exacerbate secondary injury by increasing vascular permeability and peripheral immune cell invasion following SCI (Xu et al., 1990; Sharma et al., 1993; Liu and Xu, 2010), and have been implicated in chronic neuropathic pain (Zhao et al., 2007; Buczynski et al., 2010). Treatment with a dual COX/LOX inhibitor reduced inflammation and mechanical hypersensitivity following SCI (Dulin et al., 2013).

SCI is associated with upregulation/increased activity of multiple PLA2 isoforms including cytosolic PLA2 (cPLA2) and secretory PLA2 (sPLA2) (Titsworth et al., 2008; Liu et al., 2014), cyclooxygenase-2 (COX2) (Resnick et al., 1998) and 5-lipoxygenase (5-LOX) (Genovese et al., 2005). MH has been shown to reduce cPLA2 expression following neurologic injury (Ma et al., 2010), and directly inhibit sPLA2 activity in cell-free conditions, potentially *via* binding site interference (Pruzanski et al., 1992; Dalm et al., 2010). MH has also been shown to inhibit monocyte and microglial expression of COX2 and production of pro-inflammatory prostaglandin E2 (Krady et al., 2005; Pang et al., 2012), and suppress 5-LOX expression and activation in the injured central nervous system (Chu et al., 2007, 2010). These effects of MH treatment may be partially due to inhibition of p38 MAPK pathways, as the involvement of p38 MAPK signaling pathways in cPLA2 upregulation and activation has been well established (Waterman et al., 1996; Hernández et al., 1999; Coulon et al., 2003; Kriem et al., 2005; Nito et al., 2008). Similarly, p38 MAPK has been shown to regulate LPS-induced upregulations in COX2 expression (Chen et al., 1999; Dean et al., 1999). Upregulation of 5-LOX has been associated with increased NF-κB binding in LPS-stimulated macrophages (Altavilla et al., 2009), and activation of 5-LOX is achieved by kinases downstream of p38 MAPK (Werz et al., 2000). In addition, treatment with p38 MAPK inhibitors significantly reduced inflammation-associated expression and activation of cPLA2 (Zhu et al., 2001; Kriem et al., 2005; Nito et al., 2008), COX2 (Newton et al., 2000; Nagano et al., 2002), and 5-LOX (Boden et al., 2000; Werz et al., 2000). MH likely inhibits sPLA2 isoforms predominantly *via* p38-independent direct interference with binding sites (Pruzanski et al., 1992; Dalm et al., 2010). Although associations have been made between p38 MAPK activation and sPLA2 expression/activity (Rosenson and Gelb, 2009), further investigation is warranted to determine the relationship between p38 MAPK signaling and sPLA2 expression and activation.

Regulation of glutamate-induced inflammation

Following SCI, the glutamate level rises in the extracellular space, resulting in significant tissue damage (Liu et al., 1991, 1999; McAdoo et al., 1999). NO and PLA2, known targets of MH treatment, have been shown to inhibit astrocytic glutamate reuptake (Volterra et al., 1994) and contribute to elevated glutamate levels. Glutamate has been shown to induce microglial activation and proliferation in a p38 MAPK-dependent manner, resulting in IL-1β and NO release and neuronal apoptosis, while MH treatment was shown to abrogate this effect by inhibiting microglial p38 MAPK phosphorylation (Tikka et al., 2001). These findings highlight the complex interconnections between excitotoxicity, inflammatory signaling, and oxidative stress following SCI that could potentially serve as conserved mechanisms of MH activity in multiple contexts.

Mechanisms of Anti-Oxidative Activity

Oxidative damage occurs when cells are exposed to free radicals including ROS and RNS. The reactive species destabilize cell membranes, damage organelles, proteins and nucleic acids, and trigger apoptotic or necrotic pathways resulting in cell death (Ryter et al., 2007). The widespread oxidative damage induced by the highly reactive ROS/RNS may be central in the etiology of cellular death and functional loss after SCI (Oyinbo, 2011). Following SCI, disruption of the blood-spinal cord barrier results in hemorrhage and ischemia (Mautes et al., 2000). Under ischemic conditions, many cells die due to energetic failure, buildup of acidic anaerobic metabolites, loss of ionic homeostasis and mitochondrial dysfunction, while surviving cells produce excessive reactive species upon re-oxygenation of the tissue (Kalogeris et al., 2012). Furthermore, as blood-derived leukocytes enter the spinal cord tissue *via* damaged blood vessels, they produce ROS/RNS (Trivedi et al., 2006), while blood-derived iron catalyzes lipid peroxidation reactions, yielding additional free radicals (Hall, 2011). In addition, glutamate activation of NMDA receptors increases intracellular Ca^{2+} level, which activates neuronal NO synthase (nNOS) *via* calmodulin (Conti et al., 2007). Later, microglia and astrocytes within the spinal cord become activated and upregulate iNOS and NADPH oxidase that are responsible for prolonged free radical production and tissue damage (Xu et al., 2005; Conti et al., 2007; Cooney et al., 2014). Early upregulation of nNOS after SCI has been suggested to be detrimental by increasing the oxidative stress in the injured spinal cord (Conti et al., 2007). Studies have shown that inhibition of nNOS may promote neuroprotection after SCI (Sharma et al., 2005; Sharma, 2010). In addition, inhibition of nNOS expression in motoneurons has been shown to increase their survival after spinal root avulsion (Wu et al., 2003; Sim et al., 2015). Additional sources of reactive species following SCI include glutamate-induced mitochondrial dysfunction, increased oxygen consumption and superoxide production by phago-

cytic cells, as well as release of cytosolic oxidases, lysosomes, peroxisomes, and other cell constituents from necrotic cells (Jia et al., 2012). As a result, ROS/RNS production increases significantly, contributing to inflammation and resulting in widespread damage to both the cells producing free radicals and surrounding tissue (Visavadiya et al., 2016). MH can reduce reactive species production from activated microglia and macrophages *via* previously discussed anti-inflammatory mechanisms. In addition, MH has been shown to inhibit thrombin and Zinc-induced activation of NADPH oxidase from reactive microglia, as evidenced by reduced translocation of NADPH oxidase subunit $p67^{phox}$, an indicator of active NADPH oxidase assembly required for superoxide radical production (Kumar et al., 2015). However, the mechanism of this inhibition was not reported. Additionally, MH can attenuate glutamate-induced free radical production *via* its neuroprotective effects (Garcia-Martinez et al., 2010), which will be discussed in the neuroprotection section. Moreover, MH can directly scavenge free radicals, which will be discussed in this section. The anti-oxidative activity is an important mechanism by which MH can mitigate secondary injury progression.

Direct free radical scavenging activity

In addition to inhibiting free radical production *via* its anti-inflammatory and neuroprotective effects, MH can act as a phenolic antioxidant to directly eliminate free radicals in the post-injury microenvironment. It has been shown to exhibit powerful free-radical scavenging activity (Kraus et al., 2005) due to its phenol ring structure (**Figure 2**, red box). Free radicals can remove the hydrogen atom from the phenolic hydroxyl group in MH molecules, resulting in a phenol-derived free radical that is far less reactive due to resonance stabilization and steric hindrance around the phenol group (Kraus et al., 2005). MH was found to directly scavenge free radicals including DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammoniumsalt], reduce deoxyribose degradation by Fe^{2+}/ascorbic acid/H_2O_2, and inhibit iron-induced lipid peroxidation (Kraus et al., 2005). Furthermore, studies have shown that MH can significantly inhibit lipid peroxidation after SCI (Sonmez et al., 2013; Aras et al., 2015). Treatment with MH resulted in decreased levels of malondialdehyde (MDA) (Sonmez et al., 2013; Aras et al., 2015), a byproduct of lipid peroxidation, and increased levels of glutathione (GSH), an endogenous antioxidant that neutralizes reactive species (Sonmez et al., 2013). MH treatment after SCI also led to increased activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), enzymes responsible for neutralizing free radicals (Aras et al., 2015). GSH-Px catalyzes the neutralizing reaction between reactive species and GSH, yielding a glutathione disulfide species. Thus, decreases in GSH suggest increases in reactive species concentration and subsequent GSH consumption. Similarly, increases in GSH-Px and SOD activity could suggest the presence of free enzymes not actively catalyzing neutralization reactions, because of reduced levels of reactive species. In addition to SCI, MH has been shown to reduce glutamate-induced oxidative stress in neuronal cultures (Kraus et al., 2005), attenuate oxidative stress in a model of ischemia (Morimoto et al., 2005), and protect against oxidative damage in the brains of animals challenged with chronic mild stress (Réus et al., 2015). Taken together, these data illustrate powerful anti-oxidative mechanisms by which MH can reduce secondary injury after SCI, likely through both reduced free radical production and direct free-radical scavenging. Further investigation is warranted to determine the relative contributions of each aspect of anti-oxidative activity.

Mechanisms of Neuroprotection

As we have discussed, MH can reduce the toxicity of the post-SCI environment by inhibiting the production of neurotoxic molecules through modulating inflammation and scavenging free radicals. In addition, MH can directly protect neurons and glial cells from the neurotoxic environment after SCI (Elewa et al., 2006; Plane et al., 2010). In this section, we discuss the potential mechanisms of its direct neuroprotective effects.

Protection against glutamate excitotoxicity

Glutamate excitotoxicity is one of the major secondary injury mechanisms. A number of factors contribute to elevated glutamate levels in the injured spinal cord, including enhanced presynaptic glutamate efflux from injured neurons, reduction of glutamate uptake by astrocytes, and reverse Na^+/glutamate transporter activity due to excess Na^+ ion buildup downstream of ATP synthase failure (Nishizawa, 2001; Park, 2004). Excessive glutamate activates ionotropic glutamate receptors, triggering the opening of associated ion channels and subsequent Ca^{2+} influx (Park, 2004). Activation of glutamate receptor also results in suppression of PI3K/Akt activation and subsequent p38 MAPK phosphorylation in cerebellar granule neurons (Pi et al., 2004), and increases downstream neuronal expression and activation of p38 MAPK pathway-associated pro-inflammatory and ROS-generating genes implicated in excitotoxic injury progression, including cPLA2, NO synthase, and NADPH oxidase (Dugan et al., 1995; Mark et al., 2001; Shen et al., 2007; Demaurex and Scorrano, 2009). Excessive Ca^{2+} influx also results in mitochondrial Ca^{2+} overload and has direct consequences on mitochondrial function, including uncoupling of electron transfer from ATP synthesis and resultant energy failure (Schinder et al., 1995; Kanki et al., 2004). When mitochondria become overloaded with Ca^{2+} ions, the mitochondrial permeability transition pore (mPTP) opens (Ankarcrona et al., 1995), which subsequently triggers massive depolarization of mitochondrial membranes. This results in ATP deficiency and cytosolic release of mitochondrial contents including CytC (Norenberg and Rao, 2007), a crucial mediator of both apoptotic and necrotic cell death pathways (Bobba et al., 2002; Rasola and Bernardi, 2011). In addition, increased intracellular Ca^{2+} activates calpains, a family of Ca^{2+}-dependent cysteine proteases following SCI (Ray et al., 1999, 2003,

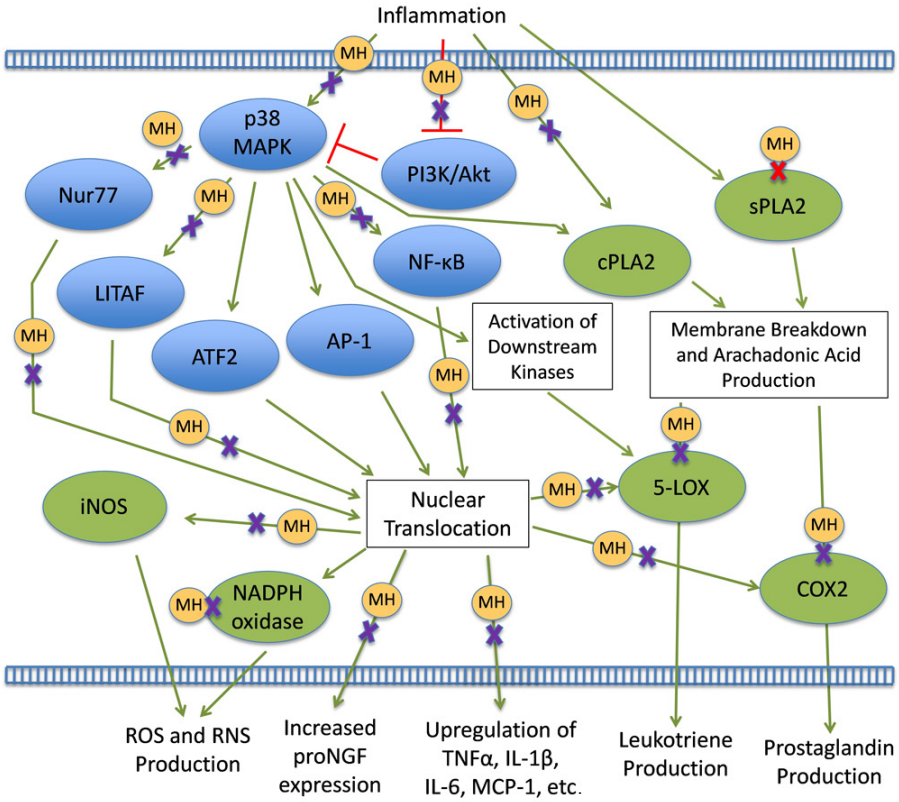

Figure 1 Inflammatory pathways involved in the anti-inflammatory action of MH.
Red x indicates direct inhibitory effect of MH. Purple x indicates that it is uncertain whether the inhibitory effect of MH is direct or indirect or both. 5-LOX: 5-Lipoxygenase; AP-1: activator protein 1; ATF2: activating transcription factor 2; COX2: cyclooxygenase-2; cPLA2: cytosolic phospholipases A2; IL-1β: interleukin-1β; iNOS: inducible nitric oxide synthase; LITAF: lipopolysaccharide-induced tumor necrosis factor-alpha factor; MCP-1: monocyte-chemoattractant protein-1; MH: minocycline hydrochloride; NADPH: nicotinamide adenine dinucleotide phosphate; NF-κB: nuclear factor kappaB; Nur77: nerve growth factor IB; p38 MAPK: p38 mitogen-activated protein kinases; PI3K: phosphoinositide 3-kinase; proNGF: proNerve Growth Factor; ROS: reactive oxygen species; TNFα: tumor necrosis factor α; sPLA2: secretory phospholipases A2.

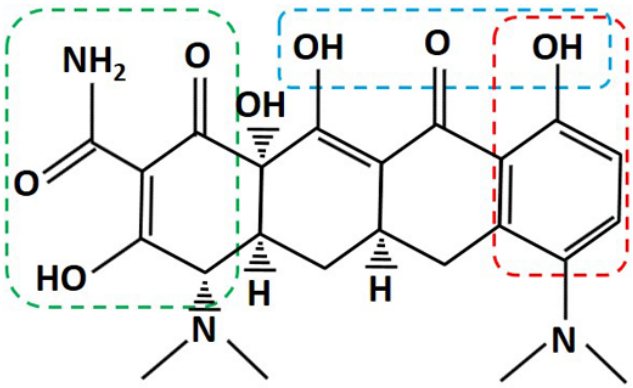

Figure 2 Chemical structure of minocycline hydrochloride (MH).
The blue box indicates metal ion chelating sites, the red box indicates the site with direct anti-oxidative activity, and the green box indicates the site with poly(ADP-ribose) polymerase-1 (PARP-1) inhibitory activity.

2011). Overactivation of calpains degrades cytoskeletal and membrane proteins, resulting in both necrotic and apoptotic death after neuronal injury (Vosler et al., 2008; Ray et al., 2011). Additionally, calpains have been suggested to act synergistically with caspase-3 activation to promote apoptosis (Ray et al., 2001; Wingrave et al., 2003). Calpains can also promote cell death through an alternative caspase-independent mechanism mediated by mitochondrial release of CytC and apoptosis-inducing factor (Lankiewicz et al., 1999; Volbracht et al., 2005).

MH has been shown to protect cultured spinal cord- and brain-derived neurons from excitotoxic insult (Tikka et al., 2001; Tikka and Koistinaho, 2001; Gonzalez et al., 2007; Garcia-Martinez et al., 2010). Multiple mechanisms of protection have been suggested and a detailed illustration of neuroprotective mechanisms of MH against excitotoxicity is presented in **Figure 3**. MH has been shown to inhibit Ca^{2+} influx through NMDA-responsive glutamate receptors (Garcia-Martinez et al., 2010). MH can chelate divalent and trivalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} (**Figure 2**) (Lambs et al., 1984; Grenier et al., 2000; Bauer et al., 2004; Chen-Roetling et al., 2009; Huang et al., 2012; Venkat et al., 2013; Wang et al., 2017). This property can theoretically reduce extracellular Ca^{2+} concentration and thereby Ca^{2+} influx. However, when cerebellar granule neurons were challenged with NMDA, MH was found to significantly reduce Ca^{2+} influx; but when cells were challenged with high K^{+} medium triggering voltage-gated Ca^{2+} channels, no effect from MH treatment was observed (Garcia-Martinez et al., 2010). Because MH chelation occurred in both contexts, this illustrates that metal ion chelation is insufficient to reduce Ca^{2+} influx. Instead, MH is likely interacting specifically with NMDA receptors. MH has been described as an NMDA receptor modulator (Chaves et al., 2009), and shown to modulate NMDA receptor signaling in hippocampal neurons (Gonzalez et al., 2007), but the exact nature of MH interaction with NMDA receptors remains poorly understood. Further investigation is warranted to elucidate a clear mechanism of action.

In addition to inhibiting Ca^{2+} influx through the cell membrane, MH was found to reduce mitochondrial Ca^{2+} uptake by slightly depolarizing mitochondria, reducing the

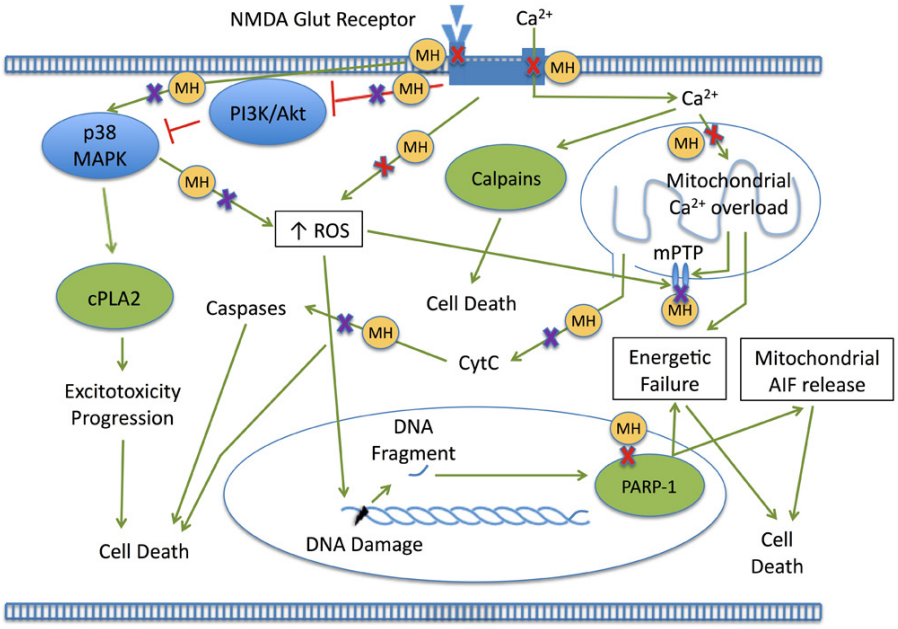

Figure 3 MH inhibits glutamate excitotoxicity in neurons.
Red x indicates direct inhibitory effect of MH. Purple x indicates that it is uncertain whether the inhibitory effect of MH is direct or indirect or both. AIF: Apoptosis inducing factor; cPLA2: cytosolic phospholipases A2; CytC: cytochrome c; MH: minocycline hydrochloride; mPTP: mitochondrial permeability transition pore; NMDA: N-methyl-D-aspartate; p38 MAPK: p38 mitogen-activated protein kinases; PARP-1: poly(ADP-ribose) polymerase-1; PI3K: phosphoinositide 3-kinase; ROS: reactive oxygen species.

electrochemical gradient required for mitochondrial Ca^{2+} uptake (Garcia-Martinez et al., 2010). The authors attributed this function to selective partial inhibition of electron transport chain complexes I and IV and modulation of the voltage-dependent anion channel (VDAC) (Garcia-Martinez et al., 2010). In addition, MH can inhibit the mitochondrial permeability transition. It is likely a result of its inhibition of mitochondrial Ca^{2+} overload and oxidative stress, prerequisites for mPTP opening (Norenberg and Rao, 2007; Webster, 2012). MH has also been shown to inhibit mitochondrial Ca^{2+} uptake in liver cells following ischemic insult (Theruvath et al., 2008; Schwartz et al., 2013), indicating a conserved mechanism of action across multiple cell types.

MH has been shown to inhibit the opening of the mPTP (Gieseler et al., 2009) and mitochondrial CytC release *in vivo* (Zhu et al., 2002; Teng et al., 2004). Although mitochondrial release of CytC is often associated with increased mitochondrial permeability, it can also occur through other mechanisms (Bossy-Wetzel et al., 1998). For example, activation of upstream proapoptotic factors also causes CytC release (Stirling et al., 2005). When CytC is released from mitochondria, it initiates pro-apoptotic caspase signaling cascades, resulting in cell death (Cai et al., 1998). MH has been shown to inhibit CytC release and improve functional outcomes in rodent models of SCI and amyotrophic lateral sclerosis (Zhu et al., 2002; Teng et al., 2004). In addition, MH has been shown to attenuate increases in caspase-1 and 3 expression *in vivo* (Chen et al., 2000; Festoff et al., 2006). Although the mechanism is not well understood, reduction in CytC release is likely a result of previously described MH activity in mitochondria, while inhibition of caspase expression could be a downstream effect of reduced CytC release.

MH can also inhibit NMDA-induced ROS production in cultured neurons (Garcia-Martinez et al., 2010). In addition to damaging proteins, lipids, and nucleic acids, ROS can directly damage mitochondria and induce mPTP opening as well (Dong et al., 2009). Reduced ROS production is likely a result of inhibition of p38 MAPK pathways, since upregulation of ROS-producing enzymes was previously shown to be dependent on p38 MAPK activation in other cell types. MH has been shown to inhibit p38 MAPK activation in neurons challenged with glutamate (Pi et al., 2004).

MH may also protect against excitotoxicity through modulation of PLA2 expression and activity, as cPLA2 has been implicated in excitotoxic progression in cultured neurons (Shen et al., 2007; Zhao et al., 2011b). In addition, activated cPLA2 was found in neurons following SCI, and implicated in injury progression (Liu et al., 2014). In neuronal cultures, cPLA2 activation has been shown to be regulated by p38 MAPK (Kriem et al., 2005), a known target of MH treatment in neurons (Pi et al., 2004). It follows that MH treatment likely reduces cPLA2 activation in neurons through inhibition of p38 MAPK pathway.

Enzymatic inhibition of PARP-1

Cell death following SCI is also associated with overactivation of poly(ADP-ribose) polymerase-1 (PARP-1) (Genovese and Cuzzocrea, 2008). PARP-1 is a nuclear enzyme implicated in DNA repair in healthy tissue, but can play a pathogenic role in response to excitotoxic insult and oxidative stress (Mandir et al., 2000; Ying et al., 2001; Du et al., 2003). PARP-1 activation is triggered by DNA damage (D'Amours et al., 1999). When the damage is mild, PARP-1 facilitates cell survival. However, severe DNA damage can induce excessive activation of PARP-1 (Wu et al., 2015), which depletes cytosolic nicotinamide adenine dinucleotide (NAD^+), resulting in ATP-deficient energy failure and massive mitochondrial depolarization (Baxter et al., 2014). PARP-1 also induces mitochondrial release of apoptosis inducing factor (AIF), which translocates to the nucleus, leading to cell death

(Wang et al., 2009). In addition, PARP-1 activation has been implicated in astrocyte activation—treatment of bacteria-stimulated astrocytes with a synthetic PARP-1 inhibitor resulted in reduced expression of IL-1β, TNFα, NO, and MCP-1 (Phulwani and Kielian, 2008). MH has been shown to effectively inhibit PARP-1 enzymatic activity under cell-free conditions in a dose-dependent manner, and to protect neurons against PARP-1 mediated death (Alano et al., 2006). The authors noted that the carboxyamide functional groups attached to aromatic rings are conserved among multiple known PARP inhibitors, and are present in MH's chemical structure, indicating a possible structural basis for its activity to inhibit PARP-1 (**Figure 2**). Additionally, MH treatment was found to reduce abnormal PARP-1 activation in a rodent diabetic retinopathy model (Wu et al., 2015). PARP-1 inhibition is one of the major neuroprotective targets in therapeutic applications. Further studies are warranted to elucidate the role of MH-mediated PARP-1 inhibition following SCI and other neurological deficits.

Inhibition of MMPs

MH has been shown to inhibit MMPs, a class of metal-ion dependent enzymes capable of digesting extracellular matrix proteins. Multiple MMPs are rapidly upregulated following SCI, and are involved in both injury and recovery processes (Zhang et al., 2011). MMP-9, one of the key MMPs involved in secondary injury progression, plays an important role in breakdown of blood-spinal cord barrier, resulting in edema and invasion of peripheral immune cells and blood-derived components. MMP-related infiltration of blood-derived factors and immune cells results in increased inflammation, oxidative stress and apoptosis after SCI (Noble et al., 2002; Zhang et al., 2011). In rat models of SCI, infiltrating leukocytes were found to be the predominant source of MMP-9 activity (de Castro et al., 2000), while treatment with an MMP-2/MMP-9 inhibitor was shown to significantly reduce barrier disruption and apoptotic cell death (Yu et al., 2008). MH has been shown to inhibit both MMP-2 and MMP-9 activities *in vitro*, with a more potent effect on MMP-9 (Paemen et al., 1996; Machado et al., 2006; Modheji et al., 2016). MH inhibition of MMP-9 has also been illustrated in animal models of stroke, cardiomyopathy, cerebral ischemia, and fragile-X syndrome (Koistinaho et al., 2005; Machado et al., 2006; Bilousova et al., 2009; Matsumoto et al., 2009). MH has been found to inhibit MMP-9 activity under cell free conditions (Paemen et al., 1996), indicating that it can directly inhibit the enzymatic activities of MMPs. It has been suggested that MH could inhibit MMP activity by interacting with Zn^{2+} ions that are critical for enzymatic activity (Golub et al., 1991; Griffin et al., 2010; Modheji et al., 2016). In a study involving multiple tetracycline derivatives, a positive correlation has been reported between tetracycline derivative affinity for Zn^{2+} ions and MMP inhibition, whereas addition of excess Zn^{2+} ions was shown to partially reverse inhibition of MMPs (Ryan et al., 2001). Thus, MH likely inhibits MMP activity *via* direct inhibition of the enzyme, by interacting with metal ion moieties.

Protection against blood-derived iron toxicity

Following SCI, blood-derived factors and cells infiltrate the spinal cord tissue *via* the disrupted blood-spinal cord barrier. Iron, a key blood component, has been shown to exert neurotoxic effects by catalyzing the formation of free radicals *via* the Fenton reaction (Winterbourn, 1995), resulting in subsequent lipid peroxidation and nucleic acid damage (Salvador et al., 2010; Núñez et al., 2012). In addition to mitigating the extent of disruption to the blood-brain barrier, MH has been shown to reduce iron neurotoxicity both *in vitro* and *in vivo* (Kraus et al., 2005; Chen-Roetling et al., 2009; Zhao et al., 2011a). MH can reduce lipid peroxidation initiated by both Fe^{2+} and Fe^{3+} which can be found in the blood (Hall, 2011; Ebrahimi et al., 2013), *via* its anti-oxidative activity (Kraus et al., 2005). In this study, MH reduced lipid peroxidation through a chelation-independent, free-radical scavenging mechanism (Kraus et al., 2005). MH has also been shown to inhibit iron neurotoxicity in cultured cortical neurons (Chen-Roetling et al., 2009). In this study, the protective effect was attributed to iron chelation, increased ferritin expression, and decreased iron-catalyzed lipid peroxidation (Chen-Roetling et al., 2009). Ferritin produced in response to iron overload can attenuate toxic iron levels, resulting in a protective reduction of iron concentration (Salvador, 2010).

Systemic administration of MH in a stroke model was shown to attenuate total serum iron levels, mitigate blood-brain barrier disruption, reduce iron-overload in the brain, and attenuate neuronal death (Zhao et al., 2011a). In this study, MH actually reduced ferritin expression in the brain, likely a result of decreased serum iron levels, BBB disruption and iron infiltration into the CNS (Zhao et al., 2011a). Because MH can chelate both Fe^{2+} and Fe^{3+} (Bauer et al., 2004; Chen-Roetling et al., 2009; Huang et al., 2012), it is possible that MH can inhibit iron-mediated toxicity partially through iron chelation *in vivo*. Primary mechanisms of direct neuroprotective action against iron toxicity, however, are likely scavenging of iron-initiated free radicals and increased ferritin expression. While in the absence of irons MH did not significantly alter ferritin production in cortical neurons, exposure to irons induced a 10-fold increase in ferritin expression, and MH and iron co-treatment induced a 17-fold increase in ferritin expression (Chen-Roetling et al., 2009). This illustrates a potential antioxidant-independent mechanism by which MH alters the neuronal response to neurotoxic iron. Further investigation is warranted to determine the mechanism by which MH can induce upregulation of neuronal ferritin expression following iron insult.

Conclusions

Minocycline exhibits potent anti-inflammatory, anti-oxidative, and neuroprotective activities after SCI. Its anti-inflammatory and neuroprotective activities are partially achieved through conserved mechanisms such as modulation of p38 MAPK and PI3K/Akt signaling pathways and inhibition of MMPs. In addition, MH directly inhibits sPLA2, which is involved in conversion of AA into prostaglandins and leukotrienes. Both lipids are potent mediators of inflammation

and secondary injury after SCI. The neuroprotective effects of MH are achieved through multiple mechanisms. Besides targeting p38 MAPK and PI3K/Akt signaling as well as MMPs, MH can also protect against glutamate exitotoxicity by diminishing Ca^{2+} influx through the NMDA receptor into neurons and reducing mitochondrial Ca^{2+} uptake. In addition, MH can exert neuroprotective effects by directly inhibiting the activities of neurotoxic molecules. For example, MH can inhibit PARP-1 enzymatic activity *via* the carboxyamide functional groups attached to its aromatic rings. Furthermore, MH is a potent antioxidant. It can directly scavenge free radicals through the phenolic hydroxyl group. Because it can target many secondary injury mechanisms, MH holds great promise for the development of an effective therapy for SCI. Further research is warranted to determine the therapeutic window, as well as optimal dose, duration, and route of MH administration to achieve maximal benefit.

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