MitoQ, a mitochondria-targeted antioxidant, delays disease progression and alleviates pathogenesis in an experimental autoimmune encephalomyelitis mouse model of multiple sclerosis

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A B S T R A C T

Oxidative stress and mitochondrial dysfunction are involved in the progression and pathogenesis of multiple sclerosis (MS). MitoQ is a mitochondria-targeted antioxidant that has a neuroprotective role in several mitochondrial and neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease. Here we sought to determine the possible effects of a systematic administration of MitoQ as a therapy, using an experimental autoimmune encephalomyelitis (EAE) mouse model. We studied the beneficial effects of MitoQ in EAE mice that mimic MS like symptoms by treating EAE mice with MitoQ and pretreated C57BL6 mice with MitoQ plus EAE induction. We found that pretreatment and treatment of EAE mice with MitoQ reduced neurological disabilities associated with EAE. We also found that both pretreatment and treatment of the EAE mice with MitoQ significantly suppressed inflammatory markers of EAE, including the inhibition of inflammatory cytokines and chemokines. MitoQ treatments reduced neuronal cell loss in the spinal cord, a factor underlying motor disability in EAE mice. The neuroprotective role of MitoQ was confirmed by a neuron-glia co-culture system designed to mimic the mechanism of MS and EAE in vitro. We found that axonal inflammation and oxidative stress are associated with impaired behavioral functions in the EAE mouse model and that treatment with MitoQ can exert protective effects on neurons and reduce axonal inflammation and oxidative stress. These protective effects are likely via multiple mechanisms, including the attenuation of the robust immune response. These results suggest that MitoQ may be a new candidate for the treatment of MS.

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1. Introduction

Multiple sclerosis (MS), an autoimmune demyelinating disease with highly disabling consequences, affects over 2.5 million people worldwide, especially young people. It is characterized by chronic inflammation and axonal loss in the central nervous system (CNS) [1–5].

The hallmark pathology in MS is inflammation of the myelin, the fatty sheath that insulates nerve cells. The inflammation denudes and scars the myelin, often resulting in permanent damage to the nerves. The cause of MS is enigmatic, although most investigators believe that the immune system attacks the white matter of brain [6]. Recent studies have found extensive demyelination in the cerebral cortex, in MS patients [7–9], even in early stages of disease progression [10]. In active gray matter lesions, activated microglia were reported to appose and ensheathe apical dendrites, neurites, and neuronal perikarya [11]. Activated microglia are a major source of cytokines and oxidizing radicals, e.g., superoxides, hydroxyl radicals, hydrogen peroxide, and nitric oxide [4,12,13], which have been found to damage cell components.

Further, the extent of lipid and DNA oxidation correlated significantly with inflammation in brain lesions of patients with MS [14]. Mechanisms of axonal degeneration that researchers have proposed in MS include cytotoxic (and subsequent) mitochondrial Ca2+ overload, the accumulation of pathologic reactive oxygen species (ROS), and mitochondrial dysfunction, any of which could lead to cell death [4,15].

To reduce myelin inflammation and subsequent denuding of myelin, researchers have turned to immunomodulatory or immunosuppressive therapy [16]. However, these treatments are not always successful, and patients have had severe, adverse effects. IFN-β and glatiramer have shown therapeutic benefits in about 50% of patients with MS, who experienced reduced relapse at a rate of about 30%, and a slowed progression of disability. Although these results are encouraging, especially since the advancements in treatment have all occurred within years the search for new compounds to reduce or to prevent autoimmune inflammation, without causing significant side effects, continues.

Since ROS has been identified as being involved in the pathology of MS in humans and in animal models of MS, an antioxidant therapy has been proposed to reduce ROS in patients with MS. One such therapy
is mitochondrial CoQ10 (MitoQ), a derivative of coenzyme Q10, that is conjugated to triphenylphosphonium cation [17]. MitoQ has been found to promote the uptake of CoQ10 into mitochondria in a cell and animal model of Alzheimer’s disease and in ischemia, where it was found to combat ROS originating in mitochondria [17,18]. Several researchers reported that MitoQ has an anti-oxidant role in vitro [18] and in vivo [17]. In addition, when high levels of MitoQ orally were given to wild-type mice (C57BL/6 strain) or up to 28 weeks, the mice exhibited no measurable adverse effects [19]. However, the effects of MitoQ in animal models of MS or in patients with MS have not yet been investigated.

In the current study, we studied the effects of MitoQ on the development and progression of an experimental autoimmune encephalomyelitis (EAE) mouse model, the most widely used animal model of MS. We also investigated the possible underlying mechanisms of MitoQ in the EAE mouse model.

2. Material and methods

2.1. EAE mouse model

Ten-week-old C57BL/6 mice (25 to 35 g) were purchased from Taconic Farms (New York, NY) and housed in the animal facility of the Oregon National Primate Center at Oregon Health & Science University (OHSU). We used these mice for EAE induction and MitoQ treatments. The OHSU Institutional Animal Care and Use Committee approved all procedures for animal care according to guidelines set forth by the National Institutes of Health.

We studied 4 groups of mice (10 mice in each group): 1) Normal control mice - C57BL6 strain, 2) EAE mice (vehicle treated), 3) MitoQ pretreated/EAE mice and 4) EAE mice for 1) clinical course of EAE, 2) mRNA expression of inflammatory, apoptotic and oxidative stress genes in tissues from spinal cord, and 3) immunohistochemistry of the spinal cords.

2.2. MOG induction

EAE induction was performed as described previously [20,21]. The C57BL6 mice were injected at ~10 weeks of age with 200 μg MOG peptide amino acids 35–55 in complete Freund’s adjuvant (CFA) with Mycobacterium tuberculosis H37RA. MOG peptides were mixed with the adjuvant to yield a 1 mg/ml emulsion of the antigen and of CFA. As an immunization, 2 × 100 μl emulsion was delivered subcutaneously to each mouse at two different sites in the trunk. Pertussis toxin (400 ng/mouse) was injected twice intra-peritoneal (i.p.), in 500 μl PBS at the time of the immunization and 72 h later. To understand behavioral changes in the progression of EAE in mice, we scored the behavioral deficits daily for all mice, using the following 5-point behavioral scoring scale: Grade 0, normal (no clinical signs of EAE); Grade 1, flaccid tail; Grade 2, mild hindlimb weakness (fast righting reflex); Grade 3, severe hindlimb weakness (slow righting reflex); Grade 4, hindlimb paralysis; Grade 5, hindlimb and forelimb weakness or paralysis, moribundity, or death.

2.3. Treatment of EAE mice with MitoQ

In the preventive treatment group of mice, we injected vehicle (PBS) or MitoQ (100 nmol/mouse, i.p. injection) 10 days before immunization with MOG and continued treating mice with MitoQ or vehicle for another 30 days, twice a week (total treatment days 40) (pretreatment with MitoQ). In therapeutic group of mice, we injected EAE mice with vehicle or MitoQ (100 nmol/mouse, i.p. twice a week) 14 days after we induced them with MOG, and we continued treating them with MitoQ for total 30 days (therapeutic treatment after disease onset). The duration of treatment – for pre- and post-EAE – is standard and acceptable to determine MitoQ protective effects against EAE in mice.

We determined 100 nmole per mouse i.p. injection twice per week, based on our earlier preliminary study. Briefly, we tested 2 doses of MitoQ in 2-month-old C57BL6 mice (n = 6 for each dose), one with 100 nmole per mouse i.p. and the other with 2 μmol per mouse i.p., twice per week for 3 weeks. In a higher dose (2 μmol per mouse i.p., twice per week), one mouse became sick, in contrast to the mice on low dosage of MitoQ, all of which were active and healthy for 3 weeks. Therefore, we chose 100 nmole per mouse i.p. twice per week for our experimental groups.

2.4. RNA isolation and real-time RT-PCR

Messenger RNA changes of several MS-associated genes were isolated from the spinal cord of the non-treated EAE mice and the MitoQ-treated EAE mice (Table 1). Using primer express software (Applied Biosystems, Foster City, CA), the oligonucleotide primers were designed for β-actin and GAPDH (housekeeping genes) and for MBP, CD4, C8, CD11b, CD11c, IL1, IL6, IL10, IL17, TGFβ1, TNFα, STAT3, NOS, NFκB, and INFγ. The real-time RT-PCR oligonucleotide primers were designed, using primer express and primer sequences; amplicon sizes are listed in Table 1. We measured mRNA expression of the genes mentioned above, using SYBR-Green chemistry-based quantitative real-time RT-PCR, as described in Manzczak et al. [22] and Reddy et al. [23]. Briefly, 2 μg of DNase-treated total RNA was used as starting material, to which we added 1 μl of oligo (dT), 1 μl of 10 mM dNTPs, 4 μl of 5 × first strand buffer, 2 μl of 0.1 M DTT, and 1 μl RNAse out. The reagents RNA, dT, and dNTPs were mixed first, then heated at 65 °C for 5 min, and finally chilled on ice until the remaining components were added. The samples were incubated at 42 °C for 2 min, and then 1 μl of SuperScript III (40 U/μl) was added. The samples were incubated again at 42 °C, but for 50 min, at which time the reaction was inactivated by heating the samples at 70 °C for 15 min.

<table>
<thead>
<tr>
<th>Marker</th>
<th>DNA sequence (5'-3')</th>
<th>PCR product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>Forward primer ACAGACAGACAGGGGACATT</td>
<td>56</td>
</tr>
<tr>
<td>CD4</td>
<td>Reverse primer CACCCCTGCCACGACTT</td>
<td>59</td>
</tr>
<tr>
<td>CD8</td>
<td>Reverse primer GGAGGACTGACGACGATTCT</td>
<td>54</td>
</tr>
<tr>
<td>CD11b</td>
<td>Reverse primer TGGGGGCGCTCTTCCAGCTT</td>
<td>57</td>
</tr>
<tr>
<td>IL1</td>
<td>Reverse primer CGATGCTGCACGAGAT</td>
<td>50</td>
</tr>
<tr>
<td>IL6</td>
<td>Reverse primer CCAATTCGCTCCGGCAT</td>
<td>55</td>
</tr>
<tr>
<td>IL10</td>
<td>Reverse primer TGGGACTGATCTCTCCTGCA</td>
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</tr>
<tr>
<td>IL17</td>
<td>Reverse primer CGGTCGCAGCTCAGGA</td>
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</tr>
<tr>
<td>TGFβ1</td>
<td>Reverse primer TTGGAGTTGAGTATCTCTTGTC</td>
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</tr>
<tr>
<td>TNFα</td>
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</tr>
<tr>
<td>STAT3</td>
<td>Reverse primer GTGGAGAATGAGTCTA</td>
<td>55</td>
</tr>
<tr>
<td>NOS</td>
<td>Reverse primer GCCAGCAGGCCCCACAT</td>
<td>58</td>
</tr>
<tr>
<td>NFκB</td>
<td>Reverse primer GCCCTGAGCTTCCAGTTC</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Reverse primer ACAAGCAGGGGACATT</td>
<td>59</td>
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</tbody>
</table>
The Ct-values of β-actin and GAPDH were tested to determine the unregulated endogenous reference gene in experimental animals and control animals. The Ct-value, described in Manczak et al. [22] and Reddy et al. [23], is an important quantitative parameter in real-time PCR analysis. All RT-PCR reactions were carried out in triplicate and with no template control. The mRNA transcript level was normalized against β-actin and GAPDH at each dilution. The standard curve was the normalized mRNA transcript level, plotted against the log-value of the input cDNA concentration at each dilution. Briefly, the comparative CT method involved averaging triplicate samples, which were taken as the Ct values for β-actin, GAPDH, and mitochondrial genes. β-actin normalization was used because β-actin and Ct values were similar for mitochondrial structural genes, and also it allows us to avoid the values of GAPDH. The ΔCt-value was obtained by subtracting the average β-actin Ct value from the average Ct-value for the mitochondrial structural genes. The ΔCt of the controls was used as the calibrator. The fold change was calculated according to the formula 2^ΔΔCt, where ΔΔCt is the difference between ΔCt and the ΔCt calibrator value.

2.5. Histochemistry and quantification of inflammatory responses

To determine the protective effects of MitoQ in microglial activity and oxidative stress, we used immunohistochemistry and immunofluorescence analyses of several selective cytokine markers that are differentially expressed in EAE mice. All mice that were used to examine behavioral analyses were sacrificed by cervical dislocation, and then the spinal cord was frozen quickly and histological examinations were conducted in the spinal cord. Briefly, we fixed the fresh-frozen tissues by dipping them into a 4% paraformaldehyde solution for 10 min at room temperature. The sections were incubated overnight at room temperature with cytokine antibodies. Details of antibodies are given in Table 2. On the next day, the sections were incubated with the secondary antibody conjugated with HRP or biotin-labeled secondary antibody for 1 h. Spinal cord sections were incubated with tyramide-labeled or streptavidin-conjugated fluorescent dye, Alexa 488 (green) (Molecular Probes, Eugene, OR). Photographs of spinal cord were taken with a fluorescence microscope.

To quantify the immunoreactivity of microglial markers Iba1 and IL6, and neuronal marker NeuN for each animal, ten sections from each mouse of the spinal cord were stained (as described above), and the immunoreactive signals were quantified. From immunostained sections, several photographs were taken at 10 × and 40 × (the original magnification) of the spinal cord. Positive immunofluorescence fluorescence signal intensity of microglia was measured using the red-green and blue (RGB) method, as described in Manczak et al. [22] and Reddy et al. [24]. The signal intensity of immunoreactivity for several randomly selected images of each mouse was quantified, and statistical significance was assessed for each marker in the EAE mice, MitoQ-pretreated, MitoQ-treated (therapeutic) mice, and normal control mice.

2.6. Co-culture of cortical neurons and microglia

Cortical neurons from wild-type mice (C57BL6; embryonic day 18 [E18]) were plated at 1 × 10^5 cells/well on poly-o-lysine-coated 24-well plates in a neurobasal medium (Invitrogen, San Diego, CA, USA) containing a supplement of 2% B-27 and 0.6 mM L-glutamine. The cells were cultured in humidified air of 5% CO2 [21,25,26]. Microglia from the BV2 cell line (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. The cells were then cultured in humidified air containing 5% CO2. Twenty-four hours after the plating, MitoQ (40 nM final concentration) was added to the neuronal culture from the treatment groups. After another 24 h, microglia (1 × 10^5 cells/well) were seeded with primary neuronal cultures. At this point, the medium containing MitoQ was removed from the “pretreatment” plates and stimulated with 1 μg/ml lipopolysaccharide for 60 h. Cells were subjected to a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or fixed with 4% paraformaldehyde for 20 min and stained with a NeuN/p3-tubulin antibody (Covance, Princeton, NJ, USA; 1:500) and a proper secondary antibody. The numbers of NeuN/p3-tubulin-positive cells were quantified in triplicate in three independent experiments.

2.7. Statistical analysis

Data were expressed as the mean ± SEM, except where otherwise indicated. Data were compared using ANOVA and Student’s t-test with SigmaStat 3.5 software; *P < 0.05 was considered statistically significant.

3. Results

3.1. Reduction of neurological disability in the MitoQ-treated EAE mice

To determine the possible role and mechanisms of MitoQ therapy administered to an EAE mouse model of MS, we first examined whether MitoQ affects behavioral deficits associated with a chronic form of EAE: forelimb and hindlimb paralysis. We followed a standard procedure to induce EAE by immunizing wild-type (C57BL6) mice with MOG.

Consistent with previous reports [20,21,27,28], our vehicle-treated EAE mice developed a typical course of EAE neurological disability, which became obvious around two weeks after immunization. The disabilities persisted for at least 8 weeks (Fig. 1). However, our EAE mice that were pretreated with MitoQ showed a modest but statistically significant delay in the onset of behavioral deficits (Fig. 1A), as well as an attenuation of symptoms over the course of EAE development (Fig. 1A, P < 0.05). Interestingly, EAE mice that received MitoQ treatment (after day 12 i.p.) had received significantly reduced scores for their symptoms (Fig. 1B). These findings point to a protective role for MitoQ in the EAE mouse model.

Table 2

Table 2 Summary of antibody dilutions and conditions used in the immunohistochemistry/immunofluorescence analysis in EAE mouse. The C-values of β-actin and GAPDH were tested to determine... of MitoQ-treatment, MitoQ-treated, MitoQ-treated (therapeutic) mice, and normal control mice.
3.2. MitoQ treatment decreases inflammation in the spinal cords of EAE mice

To determine the effects of MitoQ on EAE mice, we prepared RNA from spinal cords of control C57BL6 mice, MitoQ + EAE mice, and EAE + MitoQ mice. We compared gene expression levels, using real-time RT-PCR analysis, and we measured mRNA levels of genes that are associated with central nervous system inflammation, including MBP, CD4, CD8, CD11b, CD11c, IL1, IL6, IL10, IL17, TGFβ, TNFα, STAT3, NOS, NFκB, and INFγ.

3.2.1. MBP (myelin basic protein)

Comparing mRNA levels of MBP in EAE-induced C57BL6 mice with C57BL6 mice, we found mRNA expression levels decreased in MBP by 2.7 fold. However, mRNA expression levels were decreased in the MitoQ + EAE mice and EAE + MitoQ-treated mice relative to the C57BL6 mice (Table 3). These findings suggest that MitoQ treatment increases MBP expression in EAE mice.

3.2.2. CD4, CD8 and CD11b

As shown in Table 3, mRNA analysis of the spinal cords of EAE mice compared to the spinal cords of C57BL6 mice showed mRNA expressions levels increased in CD4 by 2.8 fold, in CD8 by 2.5 fold, and in CD11b by 2.6 fold, indicating that EAE induce enhance inflammatory response genes. In contrast, in the MitoQ-pretreated plus EAE-induced C57BL6 mice relative to the C57BL6 mice, mRNA levels were decreased in CD4 by 2.4 fold, in CD8 by 1.7 fold, and in CD11b by 6.5 fold. Similarly,

Table 3

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control mice versus EAE mice, MitoQ pretreated + EAE mice</th>
<th>EAE mice treated with MitoQ relative to control, wild-type mice</th>
</tr>
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<tbody>
<tr>
<td>MBP</td>
<td>-2.7</td>
<td>-1.3</td>
</tr>
<tr>
<td>CD4</td>
<td>2.8</td>
<td>-2.4</td>
</tr>
<tr>
<td>CD8</td>
<td>2.5</td>
<td>-1.7</td>
</tr>
<tr>
<td>CD11b</td>
<td>2.6</td>
<td>-6.5</td>
</tr>
<tr>
<td>CD11c</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>IL1</td>
<td>3.0</td>
<td>-5.4</td>
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<td>IL6</td>
<td>2.6</td>
<td>-5.1</td>
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<tr>
<td>IL10</td>
<td>1.8</td>
<td>-6.4</td>
</tr>
<tr>
<td>IL17</td>
<td>1.8</td>
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<td>TGFβ</td>
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<td>TNFα</td>
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</tr>
<tr>
<td>STAT3</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>NOS</td>
<td>2.8</td>
<td>-8.3</td>
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</tr>
<tr>
<td>INFγ</td>
<td>6.5</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Fig. 1. MitoQ treatments modestly delayed the onset and significantly attenuated the behavioral deficits of EAE. EAE was induced, disease activity was monitored daily, and the mean EAE score was calculated for vehicle-treated (filled squares, n = 10), Mito-Q pretreated, and Mito-Q EAE mice and normal control mice (filled diamonds, without MOG immunization, n = 10). The results shown are representative of 3 separate experiments.
in the MitoQ therapeutic mice mRNA levels were reduced in CD4 by 3.0 fold, in CD8 by 5.7 fold, and in CD11b by 5.2 fold (Table 3). These findings suggest that MitoQ markedly reduces inflammatory markers in the spinal cords of EAE mice.

3.2.3. CD11c
mRNA levels of CD11c did not change in the EAE-induced mice relative to the C57BL6 mice (the difference was 1.3 fold) and also MitoQ + EAE mice (the difference is 1.2 fold) and EAE + MitoQ mice (the difference is 1.3) relative to C57BL6 mice (Table 3).

3.2.4. IL1, IL6, IL10 and IL17
As shown in Table 3, in the spinal cords of EAE-induced C57BL6 mice compared to the spinal cords of C57BL6 mice, mRNA expressions levels were increased in IL1 by 3.0 fold, in IL6 by 2.6 fold, in IL10 by 1.8 fold, and in IL17 by 1.8 fold, suggesting that the inducement of EAE enhances inflammatory-response genes (Table 3). However, in the MitoQ-treated plus EAE-induced C57BL6 mice relative to C57BL6 mice, mRNA levels were decreased in IL1 by 5.4 fold, IL6 by 5.1 fold, IL10 by 6.4 fold, and IL17 by 2.5 fold, similarly, mRNA levels in the therapeutic mice (EAE + MitoQ) were reduced in IL1 by 6.0 fold, IL6 by 6.0 fold, IL10 by 10.2 fold, and IL17 by 7.4 fold. These findings indicate that MitoQ can markedly reduce inflammatory markers in the spinal cords of C57BL6 mice with EAE.

3.2.5. TNFα
Research in MS has revealed that TNFα is elevated in MS brains and in the spinal cords of EAE mice. To determine the effects of MitoQ on EAE mice, we studied mRNA levels in C57BL6 mice, MitoQ + EAE mice, and EAE + MitoQ treated mice. As shown in Table 3, TNFα mRNA levels were upregulated by 1.7 fold in the EAE mice relative to the C57BL6 mice. However, mRNA expression was reduced in the MitoQ + EAE mice by 1.9 fold and in the EAE + MitoQ mice by 2.5 fold (Table 3). These observations suggest that MitoQ can suppress inflammation in EAE mice.

3.2.6. NOS2, NFkB
Similar to other inflammatory markers, the mRNA levels of NOS2, NFkB in the EAE mice relative to the control C57BL6 mice were upregulated in NOS2 by 2.8 fold and in NFkB by 1.4 fold. These upregulated mRNA levels were reduced for NOS by 8.3 fold in the MitoQ + EAE mice and 7.9 fold in the EAE + MitoQ mice relative to C57BL6 mice. mRNA levels of NFkB were also down-regulated in the MitoQ + EAE mice by 1.8 fold and in the EAE + MitoQ by 2.6 (Table 3).

3.2.7. STAT3
In STAT3, an anti-apoptotic gene, mRNA levels were upregulated by 1.4 fold in the EAE mice relative to the control C57BL6 mice. Unlike other inflammatory markers, STAT 3 mRNA levels were upregulated in the MitoQ + EAE mice and in the EAE + MitoQ-treated mice relative to the control C57BL6 mice (Table 3), indicating that MitoQ enhances STAT3 levels. STAT3 may have some protective, beneficial effects against EAE in mice.

3.3. Immunohistochemistry analysis—Iba1
Using immunohistochemistry analysis of spinal cord sections from all 4 groups of mice, we aimed to ascertain the treatment effects of
MitoQ on inflammation and demyelination. We used a specific marker, Iba1 antibody for immunostaining of glial cells in the spinal cord because active glial cells are the main source of inflammation factors, such as pathological cytokines and ROS. Compared to normal control C57BL/6 mice (Fig. 2A and E), EAE mice (Fig. 2B and F) showed significantly high levels of Iba1 immunoreactivity (P = 0.001), indicating strong inflammation in their spinal cords (Fig. 2). Compared to the vehicle-treated EAE mice (Fig. 2B, images B and F), both MitoQ groups – the pretreated (Fig. 2C and G; P = 0.01) and the MitoQ-treated EAE mice (Fig. 2D and H; P = 0.03) – showed significantly reduced Iba1 immunoreactivity (Fig. 2), indicating MitoQ reduced CNS inflammation.

3.4. Immunohistochemistry analysis – IL6

Interleukin-6 (IL6) plays critical roles in the pathogenesis of autoimmune diseases, including EAE [29]. Importantly, IL6-deficient mice (IL6 KO) were resistant to MOG-induced EAE [30]. Also, IL6 could cause typical or atypical EAE in such IL6 KO mice [31,32]. Further, inhibition of IL6 production alleviated pathogenesis of EAE [33]. Hence we selectively examined the protein expression for IL6 in the spinal cord. Interestingly, the changes in the RNA expression of the spinal cord IL6 (Table 3) were confirmed by immunoreactivity of IL6 (Fig. 3). Compared to normal control mice (Fig. 3A and E), EAE mice showed significantly increased levels of IL6 immunoreactivity (Fig. 3B and F; P = 0.003), indicating elevated inflammation in their spinal cords of EAE mice. Compared to EAE mice (Fig. 3B and F), both the MitoQ-pretreated EAE mice (Fig. 3C and G; P = 0.01) and MitoQ-treated EAE mice (Fig. 3D and H; P = 0.01) showed significantly reduced IL6 immunoreactivity, indicating that MitoQ suppressed inflammation in EAE mice and the densities of their white matter cells were similar to those of the normal control mice.

3.5. MitoQ protective effects against neurodegeneration in EAE mice – Immunostaining analysis of neuronal marker – NeuN

In light of the profound anti-inflammatory effects that appeared to be induced by MitoQ treatments of EAE mice (Table 2; Fig. 1), we sought to determine whether these effects correlated with the preservation of neuronal cells. Our initial histological analysis indicated that the most severe neuronal damage was most frequently in the lumbar spinal cord sections (data not shown), consistent with previous studies [27,34,35]. We did not find a significant difference between the MitoQ-pretreated and MitoQ-treated mice in terms of neuroprotection. We then focused on sections from the lumbar spinal cord of EAE, EAE-treated with MitoQ, and normal control mice, using immunohistochemistry with an antibody against NeuN.

Immunostaining results are shown in Fig. 4. We observed a significant difference in the extent of neuronal loss across these mouse groups. Within the spinal cord of EAE mice, significantly reduced positively labeled with anti-NeuN antibody (Fig. 4B and F), compared to the spinal cord neurons from the normal control mice (Fig. 4A and E). However, we found significantly increased staining of anti-NeuN in the spinal cord sections from the MitoQ-pretreated EAE mice (Fig. 4C and G) and EAE mice treated with MitoQ (Fig. 4D and H). In addition, we did not find a significant difference between the MitoQ-pretreated and the MitoQ-treated EAE mice in terms of neuroprotection. Quantification of NeuN positive cells in the spinal cord confirmed significant neuronal
loss in the vehicle-treated EAE mice compared with the normal control mice, whereas the MitoQ-pretreated mice and EAE mice treated with MitoQ had NeuN positive cell numbers that were no different from numbers in the normal control mice. These data suggest that MitoQ treatment is primarily responsible for preserving demyelinated axons and neurons in the EAE mouse model. To further understand this key role of neuroprotection, we studied an in vitro EAE mouse model of MS, in which there are only two kinds of brain cells: mouse primary cultured neurons and microglial cells.

3.6. MitoQ treatment attenuates neuronal damage in lipopolysaccharide-activated microglia in vitro

To determine whether MitoQ protects against axonal damage triggered by inflammation, we used an in vitro model of microglia-mediated neurotoxicity [21,36]. As shown in Fig. 5A, cell survival was significantly reduced when cells were exposed to lipopolysaccharide. In the exposure of these cells to lipopolysaccharide-activated microglia, MitoQ treatment prevented cell death and increased cell survival, as determined by an MTT assay. This protective effect was obvious even when MitoQ was used only as a pretreatment to neurons before neurons were co-cultured with activated microglia. Specifically, in this co-culture, the number of Tuj-1-positive cortical neurons decreased after they were exposed to lipopolysaccharide-activated microglia (Fig. 5B and 5C). The treatment of neurons with MitoQ attenuated the neurotoxicity triggered by lipopolysaccharide-activated microglia. Thus, together with the in vivo immunostaining data shown in Fig. 4, these results suggest that MitoQ therapy plays a neuroprotective role that may underlie protection against mitochondrial oxidative damage in EAE.

4. Discussion

Using an EAE mouse model of MS, we found that the pretreatment and treatment of EAE mice with MitoQ reduced axonal loss and reduced neurological disabilities associated with EAE, suggesting a previously unrealized neuroprotective role for MitoQ. MitoQ also significantly reduced neuronal inflammation and demyelination, and increased and preserved axons in EAE lesions, all of which support the possibility that MitoQ treatment can protect demyelinated axons from additional degeneration. The extent of axonal protection correlated with neurological scores from these groups supporting a critical contribution of axonal damage to EAE-associated behavioral deficits.

MitoQ consists of a lipophilic cation, triphenylphosphonium, attached to ubiquinone by a saturated 10-carbon alkyl chain. Once inside the mitochondria, ubiquinone is reduced to its active ubiquinol form by Complex II [37–40]. Mechanistically, the lipophilic cation is attached to antioxidants, such as vitamin E and coenzyme Q. These lipophilic cations attached antioxidants were preferentially taken up by mitochondria due to a charge difference between mitochondria (with a negative charge) and lipophilic cation-based antioxidants (with a positive charge). These antioxidants accumulate first in the cytoplasm of cells, due to a negative plasma membrane potential. They later enter mitochondria and accumulate several hundred fold within the mitochondrial matrix [40]. They then rapidly scavenge free radicals in the mitochondria and protect mitochondria and cells from oxidative insults. It has been shown that MitoQ has a protective role in animal and cell models of several human disease, including Parkinson’s disease [41] and Alzheimer’s disease [18,42,43]. Our current study is the first to investigate the efficacy of MitoQ in an experimental mouse model of MS.
It is important to determine a safe and tolerant dosage for experimental mouse models of MS and for humans with MS. To our knowledge, the longest-term animal study with MitoQ involved regularly feeding rats (with cardiac ischemia–reperfusion injury) with drinking water that contained a higher dose of MitoQ (500 μM) [44]. The subsequent study also treated 3xAD Tg mice with MitoQ in drinking water, but at 100 μM [43]. Similarly, Wani et al. [45] used MitoQ at 100 μmol/kg (100 nmol/g) via an intragastric method for rats. These MitoQ-treated animals exhibited beneficial effects, indicating that a dosage between 100–500 μM was safe and well-tolerated in drinking water.

The method of i.p. injection is a convenient way to deliver MitoQ in many in vivo studies; however, we have found no reports using i.p. to deliver MitoQ except for our current study. As mentioned in Methods, we used MitoQ i.p. at 100 nmol/mouse (~30 g), twice per week for several weeks in our in vivo EAE mouse study. We found the 100 nmol dosage is safe and tolerable, and our EAE mice also exhibited robust beneficial effects in terms of the reduction of inflammation and neuronal loss in their spinal cords.

The cause and the pathology of MS, especially triggers that lead to axonal destruction, are still unclear. Axonal damage could result from cytotoxic T lymphocytes or soluble inflammatory mediators directly launching an immunologic attack on axons [46], or MS-related axonal damage could be a secondary event to inflammatory demyelination [47]. Genetic weakness, mitochondria dysfunction,
oxidative stress, and ion imbalance may also play an important role in MS pathogenesis [4].

Microglia are major source of ROS and inflammatory cytokines, as mentioned above. Notably, ROS is required for the phagocytosis of myelin by macrophages/microglia. Further, increased ROS has been found to be a novel therapeutic strategy that results in reduced neuroinflammation in MS [48,49]. Further, mitochondria are the main source of ROS; hence, mitochondria-targeted antioxidants, such as MitoQ, are promising candidates for the treatment of diseases involving neuroinflammation, such as MS.

In the study reported here, MitoQ therapy reduced axonal degeneration in the EAE model, suggesting this axonal damage may be mecha-
nistically related to oxidative damage found in the CNS of patients with MS. Thus, in EAE and MS lesions, demyelinated axons, no matter their cause, may become vulnerable to damage by such inflammatory mediators as proteolytic enzymes, cytokines, free radicals, and oxidative products that result from activated immune and glial cells, as proposed previously [50,51]. Therefore, a treatment that effectively changes this inflammatory pathology may hold therapeutic promise for patients with MS.

How does MitoQ protect axons from degeneration? Our previous results [18] indicated that the addition of MitoQ in a cultured neuroblastoma cells that were treated with an amyloid beta peptide decreased free radicals and increased the energy metabolism of neurons, ultimately leading to neuronal survival. In the current study, we also found that MitoQ attenuated neuronal toxicity that was induced by lipopolysaccharide-activated microglia, consistent with recent observations that mitochondrial dysfunction and energy failure may be a cause of axonal degeneration in patients with MS [4,52,53]. However, increased mitochondrial content has been found in remyelinated axons [54]. Thus, both pretreatment and treatment administration of MitoQ could improve the local bioenergetics of the affected neurons in EAE mice and protect axons from degeneration. Interestingly, some pathological cytokines, such as IL6 and infiltrated microglial cells, were found to be decreased in the MitoQ treated EAE mice. In agreement with our study, treatment of rats with MitoQ reduced IL6 and oxidative stress, and improved mitochondrial function [55]. In light of ample evidence suggesting that axonal degeneration could trigger responses of microglia (and microphage) [56], axonal degeneration may lead to microglial infiltration and activation, which may also affect the extent of axonal loss in EAE models.

Efforts have been made to research the possible neuroprotective effects of a variety of agents, including cytokines, growth factors, and blockers of voltage-gated sodium channels, in EAE models [50,51]. Our current results suggest that MitoQ is a novel, potential therapy that may protect against oxidative degeneration, thus preventing axonal damage in EAE models and, ultimately, in patients with MS. The observed correlations between MitoQ and the increase in MBP levels, the prevention of axonal and neuronal damage, and the alleviation of behavioral deficits, in particular, may prevent the decline of ATP levels in neuronal cells from degeneratively diseased animals [17,18,57]. Further, MitoQ may also confer a profound, protective effect on axonal and neuronal degeneration in EAE models. Although we cannot rule out other possibilities, the protective effects of MitoQ on neurons and other cells, perhaps, oligodendrocytes, reflect the influence of different oxidant–ATP levels resulting from MitoQ treatment. The extent of this influence may relate to the sensitivities that neurons and non-
neuronal cells may have to the cellular ATP levels.

MitoQ can readily cross the blood–brain barrier, and plasma and mitochondria membranes [17], making MitoQ additionally attractive as a promising neuroprotective treatment for patients with MS. Further, we found that MitoQ regulates some key inflammatory-associated genes, including NOS in the CNS of EAE mice. Better understanding the homeostatic regulation of neuronal oxidant–ATP levels may allow us to design ways to further enhance the protective effects of MitoQ for EAE, MS, and other diseases associated with axonal degeneration.

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References


