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Research report

Methylene blue improves streptozotocin-induced memory deficit by restoring mitochondrial function in rats

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ABSTRACT

The pathogenesis of Alzheimer's disease (AD) is well documented to involve mitochondrial dysfunction which causes subsequent oxidative stress and energy metabolic failure in hippocampus. Methylene blue (MB) has been implicated to be neuroprotective in a variety of neurodegenerative diseases by restoring mitochondrial function. The present work was to examine if MB was able to improve streptozotocin (STZ)-induced Alzheimer's type dementia in a rat model by attenuating mitochondrial dysfunction-derived oxidative stress and ATP synthesis decline. MB was administrated at a dose of 0.5 mg/kg/day for consecutive 7 days after bilateral STZ intracerebroventricular (ICV) injection (2.5 mg/kg). We first demonstrated that MB treatment significantly ameliorated STZ-induced hippocampus-dependent memory loss in passive avoidance test. We also found that MB has the properties to preserve neuron survival and attenuate neuronal degeneration in hippocampus CA1 region after STZ injection. In addition, oxidative stress was subsequently evaluated by measuring the content of lipid peroxidation products malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Importantly, results from our study showed a remarkable suppression of MB treatment on both MDA production and 4-HNE immunoactivity. Finally, energy metabolism in CA1 region was examined by detecting mitochondrial cytochrome *c* oxidase (CCO) activity and the resultant ATP production. Of significant interest, our result displayed a robust facilitation of MB on CCO activity and the consequent ATP synthesis. The current study indicates that MB may be a promising therapeutic agent targeting oxidative damage and ATP synthesis failure during AD progression.

1. Introduction

Alzheimer's disease (AD) is a progressive brain disorder hallmarked by extracellular amyloid β (Aβ) deposit, intracellular neurofibrillary tangles and mitochondrial dysfunction, which leads to irreversible memory deficit, abnormal behavior and personality change (Alzheimer's, 2015; Serrano-Pozo et al., 2011). It's estimated that over 5% elderly population over 65 years today is influenced by AD, making it gradually become a global epidemic (Gascon and Gao, 2012). Compelling evidence has indicated that mitochondrial dysfunction plays a critical role in AD pathophysiological progression (Swerdlow et al., 2010; Swerdlow, 2011). Mitochondrial dysfunction involves the alterations of mitochondrial electron transfer chain (ETC) complex activities and oxidative stress, and leads to the final neuron apoptosis. Accumulating studies also suggested that Aβ deposits facilitate neuronal vulnerability to mitochondrial dysfunction through ETC damage and oxidative stress (Calkins and Reddy, 2011; Reddy and Beal, 2008).

It's well documented that excessive production of reactive oxygen species (ROS) is the primary contributor to brain damage in AD pathology (Moreira et al., 2010; von Bernhardi et al., 2015). ROS is mainly generated from mitochondrial ETC and NADPH oxidase, among which mitochondrial ETC is thought to be the most important cellular source. In oxidative phosphorylation mechanism,

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-protein; ANOVA, one-way analysis of variance; BSA, bovine serum albumin; CCO, cytochrome *c* oxidase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ETC, electron transfer chain; ELISA, enzyme-linked immunosorbent assay; F-Jade B, Fluoro-Jade B; ICV, intracerebroventricular; MB, methylene blue; MDA, malondialdehyde; PFA, paraformaldehyde; ROS, reactive oxygen species; RLU, relative light units; TBA, thiobarbituric acid; STZ, streptozotocin; 4-HNE, 4-hydroxynonenal.

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electrons are transferred through ETC complex I–IV to the final oxygen molecules. However, a small portion of electrons randomly leaked from complex I to complex III will react with oxygen molecules, which leads to the unavoidable ROS generation (Moreira et al., 2009; Wallace, 2005). The imbalanced redox status between generation and detoxification of free radicals following mitochondrial dysfunction causes the consequent oxidative stress. ROS overproduction can potentially damage various cellular components, including nucleic acid (DNA, RNA), protein, and lipids, and exacerbate the AD pathological process. Metabolism decline is another crucial pathological character during AD progression (Price et al., 2009). As the main organelles in neurons, mitochondria function as the powerhouse in almost all the cells. According to the mitochondrial cascade hypothesis, the activities of mitochondrial ETC complexes, especially the regulating enzyme cytochrome *c* oxidase, are significantly decreased following mitochondrial dysfunction in AD, which results in the consequent ATP biosynthesis decline.

Streptozotocin (STZ) is a glucosamine–nitrosurea compound generally used to establish diabetes model in experimental animals due to its selective impairment to insulin signal pathway (Lester-Coll et al., 2006; Plaschke and Hoyer, 1993). Accumulating studies also demonstrate that intracerebroventricular (i.c.v) injection can induce a series of remarkable behavioral and pathological alterations mimicking AD characters in rodents, like apparent memory loss, metabolic decline, inflammation and oxidative stress (Grunblatt et al., 2007; Hoyer and Lannert, 1999). The huge similarities in pathological symptoms with those of AD patients qualify ICV-STZ injection a suitable experimental method for preclinical AD research (Agrawal et al., 2009; Ishrat et al., 2009; Santos et al., 2015; Zhao et al., 2015).

To date, a variety of strategies targeting mitochondria protection, such as ROS scavengers and ETC complex supplementation, have been widely investigated. However, due to their temporary effectiveness and unavoidable side effects, none of these methods have been successfully employed in clinical trial. Methylene blue (MB) is a heterocyclic aromatic compound that has been clinically used for a long history. Previous studies have suggested that MB can decrease amyloid plaques and neurofibrillary tangles formation, and restore mitochondrial function (Atamna and Kumar, 2010; Oz et al., 2009). Recent studies also indicated that MB can act as an alternative electron carrier rerouting electrons from NADH to cytochrome *c* oxidase, and enhance brain metabolism. This process bypasses the complex I/III blockage under pathological conditions, and will theoretically ameliorate ROS overproduction. The current study aims to examine the effect of MB on STZ-induced cognitive deficit in a rat model. Particularly, we investigated its neuroprotective effects on oxidative stress and ATP synthesis failure following mitochondrial dysfunction in hippocampal CA1 region. Result from our study should provide promising information for further investigation targeting mitochondrial protection in AD treatment.

2. Results

2.1. MB treatment significantly attenuated STZ-induced memory deficit in passive avoidance test

To examine the effect of MB treatment on STZ-induced memory deficit, passive avoidance test, a hippocampus-dependent memory reference task was performed 7 days after STZ injection with the index of step-through latency and numbers of rats staying in light over 300 s recorded. As we know, rats instinctively prefer the dark compartment. If an electric shock is given, a fear memory will be subsequently formed to delay the rats to reenter the dark compartment. The more memory deficit a rat suffers from, the longer latency time it will present. As shown in Fig. 1A, latencies of rats from different groups didn't show significant difference on the training day, indicating there were no distinct preferences to escape into the dark compartment among rats with different treatments. However, after electric shock was delivered on the probe day 24 h later, rats subjected to STZ injection showed significantly decreased latency time compared with control group, suggesting apparent memory deficit was caused after STZ insult. By contrast, rats treated with MB displayed a robust latency elevation, which demonstrated MB treatment can remarkably improve STZ-induced memory deficit. In consistent, apparently more rats from MB group were observed staying in the light compartment over 300 s than STZ treated rats, as shown in Fig. 1B.

2.2. MB treatment inhibited STZ-induced hippocampal neuronal degeneration

It's well known that hippocampal CA1 region plays a critical role in processing topological spatial information. Hippocampal neuronal degeneration was observed in a variety of neurodegenerative diseases at the early stage of pathological process. To investigate the effect of MB administration on hippocampal neuronal degeneration 10 days after STZ injection, hippocampal CA1 subregion was subjected to double staining of NeuN and F-Jade B, representative markers of neuronal nuclei and neuronal degeneration respectively. As shown in Fig. 2A, compared with sham control, drastic neuronal loss and apparently intensified F-Jade B staining was observed in CA1 pyramidal neurons from STZ rats, suggesting acute neuronal degeneration was induced by STZ injection. In contrast, MB treatment significantly preserved neuronal survival and inhibited STZ-induced neuronal degeneration by increasing NeuN staining and reducing F-Jade B intensity. Surviving neurons and F-Jade B positive neurons in typical 250 um were further quantified in graphical depiction with data expressed as fold changes versus sham control. As shown in Fig. 2B, about 50% of neurons loss and strong increase in F-Jade B positive neurons were triggered by STZ insult, which was significantly reversed by MB treatment, indicating MB can effectively curb hippocampal neuronal degeneration at early stage of AD progression.

2.3. MB treatment attenuated STZ-induced hippocampal lipid peroxidation

A full spectrum of oxidative lesions to neuronal components has been well documented from AD patients (Nunomura et al., 2001). Accumulating studies have demonstrated that lipid peroxidation is dramatically enhanced during AD pathology. As the most extensive products of lipid peroxidation in AD, MDA and 4-HNE contents in hippocampal CA1 region were selectively detected in this study to evaluate the lipid peroxidation level. MDA content was analyzed using an Elisa assay. As shown in Fig. 3A, MDA production in STZ group was significantly increased to ~250% compared with control group, whereas MB treatment effectively suppressed the MDA generation to ~150% of the control level. In consistent, 4-HNE content was further examined by immunofluorescent staining. As expected, 4-HNE intensity in STZ confocal images was robustly elevated versus sham control, which was remarkably reversed by MB administration, as shown in Fig. 3B. Immunostaining intensity above in a typical 250 um length was further quantified as relative immunoactivity in a diagram form, with data expressed as fold changes versus sham control, as shown in Fig. 3C.