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Methylene blue exerts rapid neuroprotective effects on lipopolysaccharide-induced behavioral deficits in mice

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Highlights

- LPS injection induced depression-like behaviours and memory impairments.
- Methylene blue treatment blocked LPS-induced depression-like behaviours.
- Methylene blue treatment reversed LPS-induced memory impairments.
- Peripheral HO1 level may be involved in fast-acting effects of methylene blue.

ABSTRACT

Depression is a recurrent neuropsychiatric disorder accompanied with other behavioral deficits, including memory impairment. **A few studies** have shown that methylene blue (MB) could promote cortical neurogenesis and exert neuroprotective effects on various brain diseases, including bipolar disorder. However, the potential antidepressant effects of MB have not been fully investigated. The present study was designed to investigate the effects of MB pretreatment on behavioral deficits and the underlying mechanisms in a lipopolysaccharide (LPS)-induced depression mouse model. Mice were given **saline** (5mL/kg) or MB (5, 20 mg/kg) intraperitoneally (i.p.) **30 min prior to lipopolysaccharide (LPS, 800 μg/kg, i.p.) or the following behavioral tests.** Thereafter, serum heme oxygenase 1(HO1) were determined **by** ELISA. The results showed that LPS significantly induced body weight loss **and** behavioral deficits **that included** increased floating time in the forced swimming test, increased immobility time in the tail suspension test, decreased sucrose preference in the sucrose preference test, and memory impairment in the novel object recognition (all $p < 0.05$) when compared with that of LPS-free mice. MB treatment significantly blocked most of these behavioral deficits induced by LPS when compared with that of mice in LPS-exposed groups. Furthermore, **MB pretreatment prevented the LPS-induced decrease in serum level of HO1.** These findings suggested that MB exerts rapidly neuroprotective effects in **an** LPS-induced depression mouse model, which may be involved in its regulation on the peripheral HO system.

Abbreviations:

ELISA, enzyme-linked immunosorbent assay; FST, forced Swimming Test; HO1, heme

oxygenase 1; LPS, lipopolysaccharide; MB, methylene blue; NMDA, N-methyl-D-aspartate; NOR, novel object recognition; NSF, novelty-suppressed feeding test; OFT, open field test; SPT, sucrose Preference Test; TST, tail Suspension Test.

Keywords: methylene blue; lipopolysaccharide; depression; memory impairment; Heme oxygenase

1. Introduction

Depression, one of the most devastating mental illnesses with relative higher incidence, imposes major burden on the society[1]. Most of the current antidepressants used in the clinic present significant therapeutic limitations, mainly including side effects, delayed onset and low ratio of efficiency[2]. Accumulating evidence implicates an interaction between oxidative stress, inflammation and depression as increased levels of pro-inflammatory cytokines are associated with depression-related symptoms and reversed by antidepressant treatments[3, 4]. In rodents, systemic administration of lipopolysaccharide **(LPS) could induce inflammatory responses, depression- and anxiety-like behaviors, and cognitive impairment[5, 6]. It was shown that patients with depression exerted increased levels of inflammatory markers, such as IL-6, TNF-a in both peripheral blood and special brain regions, which could be reversed by antidepressant treatment[7, 8]. More and more attention has been attracted to investigate the relationship between immune regulation and depression and to explore newly therapeutic strategies of depression[9]. Heme oxygenase 1 (HO1) is an inducible enzyme that has been shown to participate as an essential defensive mechanism for neurons exposed to oxidant challenges, being related to antioxidant defenses in certain neuropathological conditions. HO1 could modulate the production of proinflammatory mediators and exert protective effects on neuronal cells from oxidative stress and other toxicity stimulus, such as lipopolysaccharide and hypoxia in vivo and in vitro[10-12]. Recent studies showed that serum HO1 is decreased in patients with depression, and enhanced expression of HO1 in the locus coeruleus is associated with anxiolytic-like effects, highlighting that HO1 could be a candidate as a depression biomarker and should be fully investigated[5, 13, 14].**

Methylene blue (MB), a century-old drug for the treatment of malaria, has been shown to be neuroprotective against various physiological and pathological processes[15, 16]. MB treatment could improve memory retention when given pre- and/post-training in low doses in a variety of learning and memory tasks in normal animals[17-20]. MB could promote cortical

neurogenesis and ameliorate behavioral deficit after photothrombotic stroke, attenuate traumatic brain injury associated neuroinflammation [21, 22]. Most recently, Li et al. reported combination treatment with MB and hypothermia protects the ischemic brain and suppresses cognitive impairment caused by global cerebral ischemia[23]. The antidepressant effects of MB were firstly investigated by Naylor and colleagues in the 1980s. Since then, a series of clinical trials reported the potential therapeutic effects of MB on manic depressive illness, which was further demonstrated by using normal mice model [19, 24]. Increasing evidence reported that MB exerts neuroprotective activities in many kinds of brain disorders, including attenuating acute depressive like behavior, improving residual symptoms of depression and anxiety in patients with bipolar disorder[25-27].

However, to our knowledge, no experiments were carried out to further investigate the antidepressant-like effects of MB using depression animal models. In the present study, we mainly aimed to evaluate the effects of intraperitoneally (i.p.) pre-administration of MB on the behavioral deficits of mice exposed to LPS and the associated underlying molecular mechanism.

2. Materials and methods

2.1. Animals

Male ICR mice (20-25 g) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd., Beijing, China. All mice were kept in a climate controlled environment, at a consistent temperature (22 ± 2 ℃), humidity (≈ 60%), a 12-h light/dark cycle (dark at 8:00 a.m.). Mice had *ad libitum* access to food and water. All experiments were approved by the Local Committee on Animal Care and Use and Protection of the Hebei Medical University and were followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines.

2.2. Experimental design and drug treatments

Methylene blue (MB, M9140, Sigma-Aldrich Trading Co., Ltd., Shanghai, China) and lipopolysaccharide (LPS, Sigma-Rich Trading Co., Ltd., Shanghai, China) were freshly dissolved in 0.9% physiological saline and were prepared on the day of injection. **The doses of MB/LPS treatment were chosen based on previous studies and kept at a volume of 5 mL/kg of body weight[19, 28].** Mice were injected with saline or LPS (0.8 mg/kg, i.p.). Twenty-four hours after LPS or saline treatment, mice were submitted to behavioral tests subsequently **(Fig1. A)**. **To investigate the potential role of MB on LPS-induced behavioral abnormalities, saline or MB (5, 20 mg/kg, i.p.) were daily injected 30 min prior to LPS injection and subsequent behavioral tests.** The day after the last behavioral test, mice were decapitated and serum of mice was collected for ELISA assays.

2.3. Open field test

The OFT was performed to detect the locomotion activities of mice based on our and others previous descriptions [29-31]. The Plexiglas arena (20 × 20 × 20 cm) is divided into center and periphery zones. Briefly, the subject was put into the center area at the beginning and allowed to explore freely for 5 min. Total distances the mice traveled during test were recorded automatically with a computer-based video tracking system and analyzed by software (SMART v3.0.02).

2.4. Novelty-suppressed feeding test

The NSF test was carried out as described previously [29, 31]. In brief, after 24-h of food deprivation, the mice were transferred to the testing room, placed in a clean holding cage, and allowed to habituate for 30 min. The testing session was conducted in an open-field box (20 × 20 × 20 cm). A small piece of mouse chow was placed in the center of the box. Each mouse was placed in the corner of the testing arena facing the wall, and the time until the first feeding episode was recorded. Immediately after the mouse began to eat the chow, the tested animal was placed alone in a clean cage with a weighted piece of chow for 10 min. Latency to feeding and total food intake of each mouse were assessed.

2.5. Sucrose preference test

As described previously [30], a two-bottle, overnight preference test was given to all animals three days prior to the start of the stress regimen to serve as a baseline. Animals were presented with a 1% sucrose solution bottle and a water bottle at 7:00 pm, and the bottles were taken off the following evening at 7:00 pm. Sucrose and water levels were measured before and after the sucrose test and a difference score for each solution was recorded. The sucrose preference was determined by taking the amount of sucrose consumed and dividing it by the total amount of liquid consumed.

2.6. Tail suspension test

The TST was carried out according to our previous studies [30, 31]. Briefly, the mice were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The session was videotaped. Immobility latency during the first 2 min and immobility time during the last 4 min were measured. Immobility was defined as the absence of limb or body movements, except for those caused by respiration when they hung passively and completely motionless. During the test, mice were separated from each other to prevent possible visual and acoustical associations. Data were administrated and analyzed by SMART v3.0.02 software.

2.7. Forced swimming test

The FST was carried out as described previously [30, 31]. Mice were placed into a 12cm diameter × 30 cm height plastic cylinder filled to 15 cm with 23˚C – 25˚C water. This session was videotaped. Immobility latency during the first 2 min and immobility time during the last 4 min were measured. Immobility was defined as the absence of movement except motions required maintaining the animal's head above the water. Observers were blind to treatment groups. Data were administrated and analyzed by SMART v3.0.02 software.

2.8. Novel object recognition task

Novel object recognition task (NOR) was conducted based on previous studies [32, 33]. The experimental apparatus was an open-field box (20 × 20 × 20 cm) located in a dimly illuminated testing room. A video camera mounted on the chamber above the chamber was connected to a computer to collect data. Mice were first given a habituation session in which they were allowed to freely explore the empty open field for 5 min per day for 2 days. The training session was conducted 24 h after the habituation period. Mice were individually placed in the experimental apparatus facing the wall with two identical objects (objects A1 and A2) that were positioned in two adjacent corners, 5 cm from the walls. Animals were left to freely investigate the objects and open field for 5 min. Behavioral tests were conducted at 1.5 h (short term memory, STM) or 24 h (long term memory, LTM) after NOR training. During the test session, mice were allowed to explore freely in the open field for 5 min in the presence of one familiar (A) and one novel object (B). All objects presented similar textures, colors, and sizes, but had distinctive shapes. Exploration was defined as the mice sniffing the object with the nose at a distance of no more than 2 cm and/or touching it with the nose or forepaws. Sitting on the object was not considered exploration. As was the case during training, between each individual testing trial, the field and objects were thoroughly wiped down using 70% ethanol. Time spent exploring each object and the total time spent exploring both objects was recorded for all test periods. Recognition index

(%)= TN * 100/(TN + TF) (TN = the time spent exploring the novel object; TF = the time spent exploring the familiar object in the test session). To investigate MB pretreatment on LPS-induced memory impairment of mice, Mice were exposed to LPS (800 μg/kg) or **saline** (i.p.) after the second habituation. MB or **saline** was injected (i.p.) for a total four days, from the habituation day1 to the day for long term test of NOR, and 30 min prior to NOR training, LPS injection or NOR tests.

2.9. Enzyme-linked immune-sorbent assay

Enzyme-linked immune-sorbent assay (ELISA) was conducted according to the product instruction and our previous reports [30, 34]. Blood sample of mice was collected through extracting eyeballs. Blood samples were placed at ambient temperature for 20 min and centrifuged at 2000 revolutions per minutes for 20min. The serum was transferred into new tubes for ELISA analyses. The measurement of serum levels of HO1 were conducted with commercially available ELISA kits (HO1 ml001855; Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

2.10. Data analysis

The data were expressed as the mean ± SEM. The body weight data was analyzed using analysis of variance (ANOVA) with the appropriate between- and within-subjects factors. One-way ANOVA was employed to analyze the behavioral tests data and ELISA data, followed by post-hoc test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of MB pretreatment on LPS-induced body weight loss of mice

To investigate the effects of MB on LPS-induced body weight alteration, body weight of mice were assessed one time every two days (Fig 1 A). The statistical analysis included the between-subjects factor of group (Sal + Sal, Sal + LPS, MB5+LPS, MB20+LPS) and the within-subjects factor of test condition (Pre-TRE, Post-D1, Post-D3 and Post-D5). The results showed that

exposing to LPS induced body weight loss ($F_{3,32}$ = 5.196, P<0.01, group × test conditions). Post-hoc test analysis showed that LPS injection induced significant body weight loss ($F_{1,32}$ = 7.132, P<0.05), MB pretreatment at both doses had no effects on LPS-induced body weight loss (Fig 1 B).

3.2. Effects of MB pretreatment on locomotion and anxiety-like behaviors of mice exposed to LPS

NSF was provided to investigate the LPS and MB injection on anxiety-like behaviors of mice. One-way ANOVA of the related data revealed that there was no significant difference between groups [latency to feeding ($F_{3,35}$=0.149, P>0.05) (Fig 1 C) and total food intake ($F_{3,35}$=0.454, P>0.05) in the NSF (Fig 1 D)]. To exclude the potential impact of alteration of locomotion activities of mice on subsequent depression-like behavioral tests, total distance of mice was assessed using OFT paradigm. One-way ANOVA of total distance in the OFT revealed that there was no significant difference between groups ($F_{3,35}$=0.931, P>0.05) (Fig 1 E).

3.3. MB pretreatment blocked LPS-induced depression-like behaviors

To investigate the effects of MB pretreatment on LPS-induced depression-like behaviors, behavioral tests, including forced swimming test (FST), sucrose preference test (SPT) and tail suspension test (TST), were carried out. **One-way ANOVA on the FST data revealed significant differences among groups in the floating time ($F_{3,35}$=16.732, P<0.001) and latency to floating ($F_{3,35}$=4.093, P<0.05).** Post-hoc analysis showed that injection of LPS significantly increased the floating time of mice (P<0.05) when compared with that of Sal + Sal groups. MB pretreatment at both did significantly block LPS-induced increased floating time (both P<0.001) (Fig 2 A) and increased latency to floating (both P<0.01), when compared with that of Sal + LPS group (Fig 2 D). Meanwhile, the floating time of mice in MB+LPS group were significant lower than that of mice in Sal + Sal group (both P<0.01), indicating MB could exert antidepressant-like

activities even in normal condition.

One-way ANOVA of the TST data revealed that there were significant differences between groups of immobility time ($F_{3,35}$=11.575, P<0.001), but not latency to immobility ($F_{3,35}$=2.136, P>0.05). Post-hoc analysis showed that MB pretreatment at both doses significantly blocked the increased immobility time (P<0.005 and P<0.01 respectively, Fig 2 B), but had no effect on latency to immobility (P>0.05, Fig 2 D). Interestingly, MB treatment at 5 mg/kg dose significantly reduced the immobility time of mice when compared with that of Sal + Sal group (P< 0.05), which is consistent with the results from FST.

One-way ANOVA of the SPT data revealed that there was significant differences between groups of sucrose preference ($F_{3,35}$=6.628, P<0.005), but not total water intake ($F_{3,35}$=0.280, P>0.05). Post-hoc analysis indicated that injection of LPS significantly reduced sucrose preference (P<0.05, Fig 2 C), but had no effects on total water intake (P>0.05, Fig 2 F) when compared with that of the **saline** group. MB pretreatment at both doses significantly blocked LPS-induced decreased sucrose preference (P<0.05, P<0.01 respectively) in the SPT. All these results from behavioral tests indicated that mice exposed to LPS exhibited key depression-like phenotypes (i.e. ahedonia, despair), which could be attenuated by MB pretreatment, suggesting that MB could exert rapid antidepressant-like effects in the LPS-induced depression mouse model.

3.4. MB pretreatment blocked LPS-induced memory impairment in novel object recognition task

To investigate the potential role of MB in LPS-induced memory impairment of mice, novel objects recognition task was carried out as shown in Fig 3A. One-way ANOVA for STM and LTM data revealed that there was significant differences between treatment groups: short-term memory [$F_{3,26}$=3.472, P<0.05] and long-term memory [$F_{3,26}$=5.027, P<0.01], but had no significant difference between treatment groups for training [$F_{3,26}$=0.290, P>0.05]. Post-hoc analysis showed that injection of LPS significantly impaired the

recognition index of STM ($P<0.05$, Fig 3C) and LTM ($P<0.01$, Fig 3D), but had no effects on that during NOR training phrase ($P>0.05$, Fig 3B) when compared with that of mice in Sal + Sal group. MB pretreatment at 5 and 20 mg/kg dose significantly blocked the LPS-induced reduction of recognition index during the LTM ($P<0.05$, $P<0.01$ respectively), but had no effects on that of training ($P>0.05$) and STM ($P>0.05$). These data indicated that MB pretreatment could rapidly block memory impairment of mice exposed to LPS.

3.5. MB treatment blocked LPS-induced decreased serum levels of HO1

To investigate the potential mechanism associated with the effects of MB, serum levels of HO1 of mice were measured by ELISA. **One-way ANOVA** of the ELISA data revealed that there were significant differences between groups of serum level of HO1 ($F_{3,18}=5.024$, $P<0.05$). Post-hoc analysis showed that LPS injection significantly decreased the serum levels of HO1 ($P<0.05$) when compared with that of mice in Sal + Sal group. MB pretreatment at both doses significantly blocked LPS-induced reduction of serum level of HO1 (both $P< 0.01$, Fig 3E). These results indicated the peripheral HO system may be associated with the neuroprotective effects of MB in LPS-induced behavioral abnormalities.

4. Discussion

The neuroprotective effects of MB have been widely investigated using animal models and from clinic [19, 20, 35]. MB treatment could improve memory retention, promote cortical neurogenesis, ameliorate behavioral deficit after photothrombotic stroke and attenuate traumatic brain injury associated neuroinflammation [20-22]. Several studies have also reported that MB could exert antidepressant-like effects [19, 25]. No evidence was presented using depression animal models to investigate the rapid antidepressant-like effects of MB and the potential underlying mechanism, which provide large limitation for clinic use of MB. The results of this present study demonstrated that MB pretreatment could have a rapid onset to prevent LPS-induced behavioral abnormalities, including depression-like behavior and memory impairment, without effects on body weight alteration and locomotion activities of mice. Additionally, the rapid neuroprotective effects of MB were associated to its regulation on peripheral levels of HO1, which are well known as anti-inflammatory and antioxidant molecule.

Increasing evidence has highlighted a strong association between the immune system and depression [36]. Activation of the immune system through systemic administration of LPS induces time-dependent inflammatory response and behavioral alterations, some of which are similar to clinical symptoms of depression in humans. LPS-induced depression mice model has been widely used to screen for potential new antidepressant compounds and to investigate the mechanism of depression [6, 37, 38]. Shortly after LPS injection (2-6h), mice display sickness behaviors. Following 24h after LPS injection, mice display specific depression-like behavior, such as anhedonia and despair-like behaviors, without alterations in locomotion activity. The TST, FST, and SPT are validated behavioral models of depression used in rodents. In agreement with previous studies, our results demonstrated that mice treated with LPS exerted significantly sickness and behavioral abnormalities, such as

decreased body weight, reduced sucrose preference in the SPT, increased floating time and immobility time in the FST and TST, without affecting the locomotion activities of mice in the OFT. Meanwhile, LPS injection induced significant memory impairment assessed by NOR paradigm.

It is well known that current pharmacotherapies for depression in the clinic exhibit slow onset, side effects, and limited efficacy. Since most traditional antidepressants present a delayed onset of antidepressant effects and the therapeutic latency of these agents raises the risk of patient committing suicide, identification of novel fast-onset antidepressants is desirable. Recently, many studies have been carried out to screen fast-acting antidepressants. Ketamine, one of antagonists of N-methyl-D-aspartate (NMDA) receptor has been revealed to exert rapid antidepressant effects[39]. Using chronic mild stress- and olfactory bulbectomy-induced mice model, Mcmurray and colleagues reported that inhibition of GLO1, a ubiquitous cellular enzyme, exert fast acting antidepressant effects[40]. Also some traditional herbal medicine, such as Yueju, has been shown to promote a fast-onset antidepressant effect clinically and in preclinical studies[41, 42]. The antidepressant-like effects of MB have been previously described in the clinic and in rodents subjected to the TST or to the FST in normal condition [19, 25, 26]. Most recently, MB was reported to attenuate traumatic brain injury associated acute depression-like behaviors [22]. Whether the MB could produce fast-acting activities in depression mice model is still elusive. Considering the slow onset effect of classic antidepressants and to further understand the potentially rapid antidepressant-like effects of MB, LPS-induced depression animal model were carried out in the present research. Mice were injected with MB 30 min prior to LPS injection and/or prior to behavioral tests daily. Behavioral results from the present study showed that MB pretreatment significantly blocked LPS-induced depression-like behaviors and memory impairment without blockage body weight loss of mice, suggesting a fasting-onset character of MB. Notably, several previous studies also reported that both chronic MB treatment and microinjection of MB into dorsal periaqueductal gray could exert significant anxiolytic effects [19, 35, 43]. Unfortunately, results from our present study did not find the similar anxiolytic-like effects of MB in mice exposed to LPS,

assessed by OFT and NSF, which are not consistent with that of previous studies. The differential results may be due to the different MB doses, the different duration of MB treatment and/or the different conditions of animal were kept in, which should be further investigated.

The mechanisms underlying the neuroprotective effects of MB, such as preventing neuronal damage and facilitating neuronal repair, have been well investigated [16]. MB can protect against neuronal apoptosis by suppressing mitochondrial dysfunction and subsequent oxidative damage and ATP decline. Also, MB can supports neurogenesis by ameliorating neuroinflammation and promoting neurite outgrowth and synaptogenesis. MB may act via various mechanisms which include modulation of the NO-cGMP cascade, enhancement of mitochondrial respiration and antioxidant effects[16, 44]. Oxidative stress injury and immune dysregulation, especially inflammatory processes, are well accepted to be associated with symptoms of depression[45]. Previous studies have indicated that peripherally injected LPS may promote many proinflammatory cytokines releasing into the plasma and induce depression- and anxiety-like behaviors. Anti-inflammatory and anti-oxidative stress strategies have been attracted more attention to find potential new antidepressants[46, 47]. **HO1, a well-known antioxidative molecule, was reported to exert protective effects on neuronal cells from oxidative stress in vivo and in vitro and to be associated with the pathological process of depression. Meanwhile, MB could protect RAW264.7 cells from H_2O_2-induced injury through the up-regulation of HO1, providing the possibility that HO1 may be involved in the neuroprotective effects of MB[10]. Results showed that LPS injection induced significant behavioral deficits accompanied with decreased serum levels of HO1, while MB pretreatment could block the reduction of serum HO1 induced by LPS, suggesting the neuroprotective effects of MB are associated with its regulation on production of serum HO1**. However, the precise mechanism underlying the neuroprotective effects of MB are still elusive and should be further investigated.

In summary, the findings presented here demonstrate a fast-acting character of neuroprotective effects of MB in LPS-induced depression mouse

model. The neuroprotective effects of MB pretreatment may be associated with its regulation on peripheral HO system, which need to be well investigated.

Author contributions

S.H and L. S conceived and designed the experiments. Y.S, W.X, L.W and S.J performed the behavioural Tests. Y. X, S. J and G.Y analysed the behavioural data and prepared the figures. S.H and S.J conducted the ELISA assays and analysed the data. S.H, L.S and Velez de-la-Paz O wrote and/or revised the paper.

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Figure legends

Fig.1. Methylene blue pretreatment had no effects on LPS-induced body weight loss and locomotion activities in mice. (A) Experimental procedure. The mice were given daily administration (i.p.) of **saline (Sal),** methylene blue (MB, 5, 20 mg/kg) 30 min prior to LPS (800 μg/kg, i.p.) or prior to each behavioral test. Body weight of mice was assessed during the days before LPS treatment (Pre-TRE), and 1, 3, 5 days after LPS treatment (Post-D1, Post-D3, and Post-D5). MB pretreatment had no effects on body weight loss induced by LPS injection (B). Neither LPS injection nor MB pretreatment had effects on latency to feeding (C), total food intake (D) in the NSF test and total distance in the OFT (E). **P < 0.01 versus the Sal + Sal group. n = 8–10 per group. OFT, open field test; NSF, novelty suppressed feeding; SPT, sucrose preference test; FST, forced swimming test; TST, tail suspension test.

Fig.2. Methylene blue pretreatment significantly blocked LPS-induced depression-like behaviors. Methylene blue (MB) pretreatment at both doses significantly reversed LPS-induced increased floating time (A) in the FST, increased immobility time in the TST (B) and decreased sucrose preference (C) in the SPT. MB pretreatment prolonged latency to floating (D) in the FST, but had no effects on latency to immobility (E) in the TST and total intake (F) in the SPT. *P < 0.05, **P < 0.01 versus the Sal + Sal group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 and $^{\#\#\#}$P < 0.005 versus the Sal + LPS group. n = 8–10 per group.

Fig.3. Methylene blue pretreatment significantly blocked LPS-induced memory impairment and decreased serum level of HO1. (A) Experimental procedure. Memory impairment was induced by LPS injection (800 μg/kg, i.p.) and assessed by novel objects recognition. MB administration daily for a total 4 times from the day before LPS injection (D1) or 30 min prior to LPS injection (D2), prior to NOR training (D3) or to long term memory(LTM) test (D4).

Neither LPS nor MB treatment had effects on recognition index during the training phrase (B). LPS injection induced decreased recognition index during both short-term memory (STM) test (C) and long-term memory (LTM) test (D) and decreased the serum levels of HO1 (E). MB pretreatment significantly blocked LPS-induced memory impairment during the LTM test and alteration of serum level of HO1. *P < 0.05, **P < 0.01 versus the Sal + Sal group; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 versus the Sal + LPS group. n = 7–10 per group for behavioral results. n = 4–6 per group for ELISA analysis.