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# Protective effects of vitamin B12 against LPS-induced cognitive impairment through modulation of neuroinflammation, oxidative stress, and cholinergic dysfunction

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**ABSTRACT**

***Objective(s):*** Neuroinflammation is a key pathological driver of cognitive impairment in neurodegenerative diseases. The endotoxin lipopolysaccharide (LPS) is used to model neuroinflammation, memory impairment, and neurodegeneration. Vitamin B12 has been extensively studied as an anti-oxidant and anti-inflammatory compound that plays an important role in neurological health. The protective effects of vitamin B12 against LPS-induced cognitive impairment, neuroinflammation, oxidative stress, and cholinergic dysfunction were investigated.

***Materials and Methods:*** Male Wistar rats were randomly assigned to five groups (n=10 per group): Control, LPS (1 mg/kg, intraperitoneal injection), and three LPS+vitamin B12 (cyanocobalamin) groups receiving 0.25, 0.5, or 1 mg/kg of vitamin B12 by oral gavage. The Morris water maze and passive avoidance tests were conducted, and tissue samples were used to assess oxidative stress, inflammatory markers, and cholinergic activity in the animals' brains.

***Results:*** Vitamin B12 significantly improved memory in the Morris water maze and passive avoidance tests compared with the LPS group ($P$<0.001), with higher doses being more effective. Vitamin B12 also attenuated oxidative stress and the mRNA expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 ($P$<0.05-0.001). It also significantly decreased acetylcholinesterase activity ($P$<0.01-0.001), while the highest dose significantly increased the expression of muscarinic acetylcholine receptor 1 ($P$<0.01).

***Conclusion:*** These findings suggest that B12 may reduce inflammation and oxidative stress, modulate the cholinergic system, and have potential for the prevention and treatment of neuroinflammation and inflammation-induced memory impairment.

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## Introduction

Inflammation and neuroinflammation have been implicated in the pathophysiology of neurological disorders, leading to neurodegeneration (1). This neurodegeneration then causes cognitive impairment, a hallmark of Alzheimer's disease (AD) (1). In AD, neuroinflammation has been shown to be a significant contributor to cognitive impairment and memory loss (2), which is also accompanied by cholinergic dysfunction (3). Among cholinergic markers, muscarinic acetylcholine receptor 1 (CHRM1) is expressed in the hippocampus and cerebral cortex (4) and plays a key role in cognitive function, learning, and memory (5, 6). CHRM1 dysfunction has been implicated in cognitive impairment and cholinergic deficits in neurodegenerative disorders (7).

Lipopolysaccharide (LPS) is an endotoxin of Gram-negative bacteria that can start a neuroinflammatory response by binding to toll-like receptor 4 (TLR4) and increasing inflammation through several signaling pathways, resulting in dysfunction or death of neural cells (1, 2). LPS is known to cause oxidative stress (3-5), which in turn can affect the inflammatory response (6). LPS is well established to affect many inflammatory and anti-inflammatory cytokines; tumor necrosis factor (TNF)-α and interleukin (IL)-1β were increased, but IL-4 and IL-10 were decreased (7). These inflammatory processes have been shown to cause cognitive and memory impairment (8). Vitamin B12, also known as cobalamin, is an essential

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water-soluble vitamin that functions as a critical cofactor with important roles in metabolism and DNA methylation and is obtained through digestion (9, 10). Vitamin B12 deficiency has also been linked to many complications, including neurological disorders (10, 11). In addition, a link between vitamin B12 and AD has been suggested (12). Vitamin B12 has been implicated in exhibiting anti-oxidative properties (20, 21) and can scavenge reactive oxygen species (13, 14). Vitamin B12 has also been linked to inflammation and pro-inflammatory cytokines (15), and higher B12 levels are correlated with lower inflammation (25). Vitamin B12 also has anti-aging and anti-cognitive decline effects (16-18). Using a rat model of multiple sclerosis, it was shown that vitamin B12 improved learning and memory in the passive avoidance test, accompanied by activation of AKT, brain-derived neurotrophic factor (BDNF), and cyclic adenosine monophosphate (cAMP)- response element-binding protein (CREB) in the hippocampus (19). Also, in lead-exposed rats, B12 at a dose of 1 mg/kg improved performance in the object recognition test (20). In another study, cholinergic dysfunction was induced in rats by making a lesion in the nucleus basalis, and it was shown that B12 in combination with egg phosphatidylcholine improved learning and memory in the Morris water maze and increased acetylcholine concentration in the brain (21). The protective effect of vitamin B12 on rotenone-induced memory impairment has also been reported(22). Beneficial effects of B12 on learning and memory in a nicotine withdrawal model in rats were attributed to its protective effects against oxidative stress, neuroinflammation, and acetylcholinesterase (AChE) overactivity (23). All of these findings suggest the anti-oxidant and anti-inflammatory properties of vitamin B12. Still, its neuroprotective effects against LPS-induced cognitive impairment and their relationship with oxidative stress, inflammatory gene expression, and cholinergic dysfunction have not been comprehensively evaluated. Therefore, the present study aimed to investigate the effects of vitamin B12 on LPS-induced cognitive impairment by assessing behavioral performance, oxidative stress, inflammatory responses, and cholinergic function. We hypothesized that vitamin B12 administration would attenuate LPS-induced cognitive impairment by modulating oxidative stress, neuroinflammatory responses, and cholinergic function.

## Materials and Methods

### *Animal model and intervention*

Wistar rats (9-11 weeks old, 250 ± 10 g) were purchased from Mashhad University of Medical Sciences and were kept in standard conditions (temperature: 24 ± 2 °C, 12/12-hr light/dark cycles) with regular ventilation. Food and water were freely available. Methods and procedures were approved by the ethics committee of Mashhad University of Medical Sciences. Lipopolysaccharide (LPS; *Escherichia coli* serotype O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA), and vitamin B12 (cyanocobalamin) was obtained from Raha Pharmaceutical Co. (Isfahan, Iran). G*Power software was used to calculate the sample size. Considering α=0.05, power=0.90, and using the mean time in the target area (max=30, min=17, SD=8 based on previous studies), the sample size was determined to be 50. A computer-based randomization method (using the RAND function in Microsoft Excel) was used, and the animals were randomly allocated to 5 groups of 10 rats each. The control group received normal saline (0.9% NaCl) both orally and intraperitoneally (IP) as a vehicle, and the LPS group received 1 mg/kg of LPS, which was dissolved in normal saline and administered via IP injection, as well as oral administration of normal saline. The selected LPS dose was based on previous studies demonstrating reliable induction of neuroinflammation and cognitive impairment in rats (24, 25). Vitamin B12 was administered orally by gavage in 3 groups, including LPS + B12 (0.25 mg/kg), LPS + B12 (0.5 mg/kg), and LPS + B12 (1 mg/kg), which received 0.25, 0.5, and 1 mg/kg of it (orally) in addition to LPS (IP). The experimental period included 21 days. Vitamin B12 was administered during days 1-14 without LPS. From day 15, the behavioral tests were started and continued until day 21. During this period, LPS was injected daily, as in a previous study (36), 2 hr before the behavioral tests. Treatment with different doses of B12 was continued for 30 min before LPS infection during these days.

### *Morris water maze*

Spatial memory and learning were evaluated using the Morris Water Maze (MWM) apparatus, consisting of a circular tank measuring 150 cm in diameter and 60 cm in height. Water temperature was kept at 24 ± 1 °C. The tank was separated into four quadrants. A hidden platform was positioned in one quadrant and remained in the same location throughout the experiment. The platform was placed just below the water surface to prevent visual detection by the animals. In the MWM test, the learning phase spanned five consecutive days (days 16-20 of the experimental period), followed by a probe trial. During the learning period, each animal underwent 4 trials per day. In each trial, the subject began from one quadrant and was allowed 60 sec to locate the platform. When the subject successfully located the platform within 60 sec, it was permitted to remain on the platform for 20 sec. When the animal failed to locate the platform independently, it was directed to the platform and allowed to rest for 20 sec before starting the subsequent trial. The surrounding walls featured multiple black markings and symbols on a white background, serving as visual cues to help the animals remember the platform's position relative to these landmarks. Following the 5th day of testing, the platform was extracted (day 21), and subjects completed the trials of 60 sec each without the platform present. Between trials, animals were removed from the tank for a rest period before commencing the next trial. The probe trial was performed to assess the animals' retention of the platform location, as evidenced by their continued searching despite the platform's removal. All testing data were recorded via an overhead camera (26).

### *Passive avoidance*

Fear-based memory and associative learning were examined using the passive avoidance (PA) test. The apparatus consisted of two equally sized compartments (40 cm×20 cm×20 cm). One compartment featured opaque walls and was well-lit, while the other compartment remained dark. A sliding removable door separated the dark and light sections. The test was performed over a 5-day period. The initial day (day 14) served for habituation purposes, followed by administration of a mild electrical shock (2 mA, 50 Hz, for 2 sec) on the second day (day 15), with subsequent testing conducted at 3 hr (day 15), 24 hr (day 16), 48 hr (day 17), and 72 hr (day 18) post-shock. Each

**Table 1.** analysis to investigate CHRM1,TNF-α, IL-6, and IL-1β

| Biomarkers name | Sequence of primers | |
|---|---|---|
| | Forward (5' -> 3') | Reverse (5' -> 3') |
| CHRM1 | AGTCCCTCACATCCTCCGAA | TTCTTGGTGGGCCTCTTGAC |
| TNF-α | ACACACGAGACGCTGAAGTA | TTCCGGGATCCAGTGAGTTC |
| IL-6 | CCTTCTTGGGACTGATGTTGTT | TATACTGGTCTGTTGTGGGTGG |
| IL1-β | GACTTCACCATGGAACCCGT | GGAGACTGCCCATTCTCGAC |
| GAPDH | AGTGCCAGCCTCGTCTCATA | TGAACTTGCCGTGGGTAGAG |

CHRM1: Muscarinic acetylcholine receptor 1; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IL1-β: Interleukin-β

test session lasted 300 sec, during which rats were positioned in the illuminated compartment and confined there for 30 sec before the sliding door was opened, allowing free movement between compartments. During the initial shock phase, once the rat first entered the dark compartment, the sliding door was closed, and the subject received the electrical shock. The latency to enter the dark compartment, the frequency of entries into the dark compartment, and the total time spent in the dark and light compartments were recorded. The behavioral tests, including MWM and PA tests, were carried out by an experimenter who was unaware of the treatments (19).

***Oxidative stress***

Animals were anesthetized using I.P. injection of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively, I.P.). Following anesthesia, the brains were extracted and rinsed with phosphate-buffered saline (PBS). Tissue samples were weighed and homogenized with PBS prior to subsequent biochemical evaluation. Three parameters were quantified as markers of oxidative stress in the rat hippocampus: malondialdehyde (MDA), thiol content, and superoxide dismutase (SOD). MDA serves as an indicator of lipid peroxidation and was assessed by its reaction with thiobarbituric acid (TBA) (3). A solution was prepared by dissolving 0.375 g of TBA in 2 ml of hydrochloric acid (HCl), then adding 15 g of trichloroacetic acid (TCA) to bring the final solution volume to 100 ml. Once the solution was ready, 1 ml of 10% homogenate was combined with 2 ml of the TBA-TCA-HCl mixture and incubated in boiling water. After cooling, the mixture was centrifuged, and absorbance was measured at 535 nm. MDA concentration was determined using the formula: C (M)=A/1.65 × $10^5$.

Tissue thiol content was determined in homogenates following the Ellman method. Supernatants were incubated in 1 ml Tris-ethylenediaminetetraacetic acid (EDTA) buffer (pH=8.6) with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). 50 µl of homogenate and 1 ml of Tris-EDTA buffer were combined, and absorbance was measured at 412 nm (3). Subsequently, 20 µl of DTNB reagent (10 mM in methanol) was added, and absorbance was recorded after 15 min. DTNB served as the blank. Thiol concentration was calculated using the formula: Total thiol concentration (mM)=($A_2$ - $A_1$ - B) × 1.07/0.05 × 13.6

SOD activity was assessed using a colorimetric assay described by Madesh and Balasubramanian, performed in 96-well microplates. SOD was generated via auto-oxidation of pyrogallol with subsequent inhibition of 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) reduction to formazan. Upon addition of dimethyl sulfoxide (DMSO), the reaction was terminated, and the solution color stabilized. Tissue homogenate was introduced into the wells at room temperature for 5 min. Absorbance was measured at 570 nm. The results presented in this study are expressed as units per gram of tissue (26).

***Acetylcholinesterase (AChE)***

Activity of AChE was assessed in the brain cortex and hippocampus using acetylthiocholine (ATC) as a substrate. The reaction mixture contained 40 µl of brain homogenate (10%), 2.6 ml of phosphate buffer (0.1 M, pH 8.0), and 100 µl of DTNB. The reaction mixture was mixed and placed in the spectrophotometer. Once the reaction was stable, the absorbance was recorded at 412 nm for the basal reading. The reaction was started by adding 20 µl of ATC to this mixture, and the change in absorbance was recorded at time zero and after 10 min at 25 °C. The activity of AChE was expressed as m moles per min per gram of brain (26).

***RNA extraction and quantitative real-time PCR***

Total RNA was extracted from homogenized hippocampal tissue (Norgen, Iran) according to the manufacturer's instructions. The concentration and purity of the isolated RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was subsequently synthesized from equal amounts of total RNA using a cDNA synthesis kit (Yektatajhiz Co., Tehran, Iran) following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the SYBR Green master mix (SMOBIO, Taiwan) on a Roche real-time thermal cycler (Roche Diagnostics, Germany) (26).

***Statistical analysis***

The Kolmogorov–Smirnov test was used to assess the normality of the data. Levene's test was also used to evaluate homogeneity. The results of the MWM during the 5 days of learning were analyzed using repeated-measures analysis of variance (ANOVA) (treatment and days were considered as between-subject and within-subject factors, respectively) followed by the Bonferroni multiple comparison test. The data from the probe test and biochemical data were analyzed using one-way ANOVA followed by Tukey's *post hoc* comparison test. For the data that had a non-normal distribution, Kruskal-Wallis followed by Dunn's *post hoc* test was used. The data were presented as mean±standard error of the mean (SEM) or median and interquartile range (IQR). Differences were considered statistically significant when $P<0.05$.

## Results

***Morris water maze results***

The Morris water maze was conducted for 5 days straight,

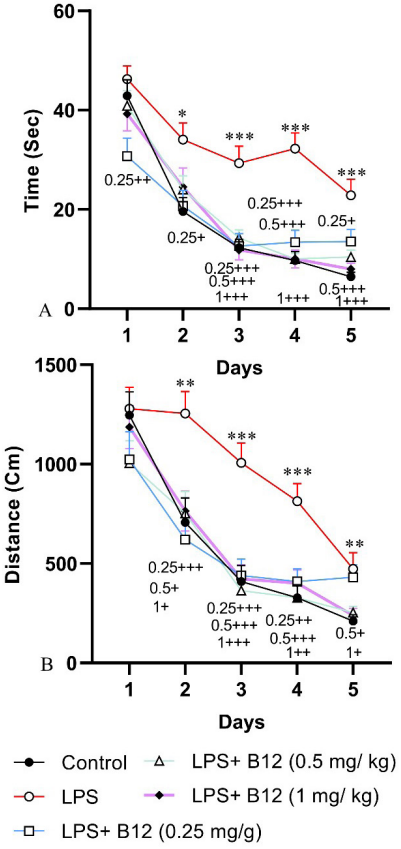


**Figure 1.** Escape latency (A) and distance traveled (B) to reach the platform in the Morris water maze in rat groups
Data are presented as mean±SEM (n=10 per group). Statistical significance: *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs control group; +$P$<0.05, ++$P$<0.01, +++$P$<0.001 vs LPS group. The numbers 0.2, 0.5, and 1 show the doses, including 0.25, 0.5, and 1 mg/kg of B12.
B12: Vitamin B12; SEM: Standard error of the mean; LPS: Lipopolysaccharide

followed by a probe day. The time spent while finding the platform and the distance traveled were recorded. Analysis of time spent to find the platform with repeated measures ANOVA showed significant main effect of treatment ($F(4,195)=19.63$, $P$<0.001), day (with Greenhouse- Geisser correction: $F(3.67, 717.23)=104.10$, $P$<0.001), and treatment × day interaction (with Greenhouse- Geisser correction: $F(14.71, 717.23)=2.26$, $P$<0.01). Bonferroni pairwise comparison showed that the difference in the time spent to find the platform was not significant between LPS and the control groups on the first day. The difference between LPS and control was significant on the second day ($P$<0.05) and also on the next 3 days ($P$<0.001 for all 3 days) (Figure 1A). As for the treatment groups, on the first and second days, the only group that was significantly better than LPS was the 0.25 mg dose ($P$<0.01 and $P$<0.05, respectively). On the third day, every dose considerably reduced the time spent to find the platform ($P$<0.001). This trend continued on the 4th day ($P$<0.001 for all doses). On the last day, the 0.25 ($P$<0.05), 0.5 ($P$<0.001), and 1 mg/kg ($P$<0.001) doses were still significantly different compared to LPS (Figure 1A). Analysis of distance traveled to find the platform with repeated measures ANOVA showed significant main effect of treatment ($F(4,195)=14.17$, $P$<0.001), day (with Greenhouse-Geisser correction: $F(3.47, 678.45)=72.81$; $P$<0.001), and treatment×day interaction (with Greenhouse- Geisser correction: $F(13.91, 678.45)=1.78$, $P$<0.05). Furthermore, Bonferroni pairwise comparison showed that, compared to the other groups, LPS traveled a substantially longer distance in the tank before resting on the platform (Figure 1B). The difference between LPS and control was significant every day except for the initial day of the trial ($P$<0.01 for days 2 and 5, $P$<0.001 for days 3 and 4). Animals that received a 0.25 mg dose traveled significantly shorter distances compared to LPS on the 2nd ($P$<0.001), 3rd ($P$<0.001), and 4th ($P$<0.01) days and were not different compared to LPS on the 1st and last days of the test. On the other hand, the 0.5 mg and 1 mg doses were significant every day except for the first day with varying levels of significance (0.5 mg: $P$<0.05 on the second day, $P$<0.001 on the third day, $P$<0.001 on the fourth, and $P$<0.05 on the fifth day, 1 mg dose: $P$<0.05 for the second day, $P$<0.001 for the third day, $P$<0.01 for the fourth day, and $P$<0.05 for the fifth day) (Figure 1B).

After the 5th day, a probe test was conducted, and time and distance were recorded for each subject. The results revealed that there was a significant difference between the groups in the traveling time and distance in the target area in the probe trial ($F(4, 195) = 11.78$, $P$<0.00 for traveling time and $F(4, 195) = 8.76$, $P$<0.001 for traveling distance). The results also showed that in the LPS group, the amount of time and distance in the target quadrant was significantly less than in the control group ($P$<.001) (Figures 2A and 2B). After the treatment, the difference between the LPS group and LPS + B12 (0.25 mg/kg) and LPS + B12 (0.5 mg/kg) was not significant. Still, the highest dose of B12 increased the time and distance in the target area significantly compared to the LPS group ($P$<0.001). There was also a significant difference

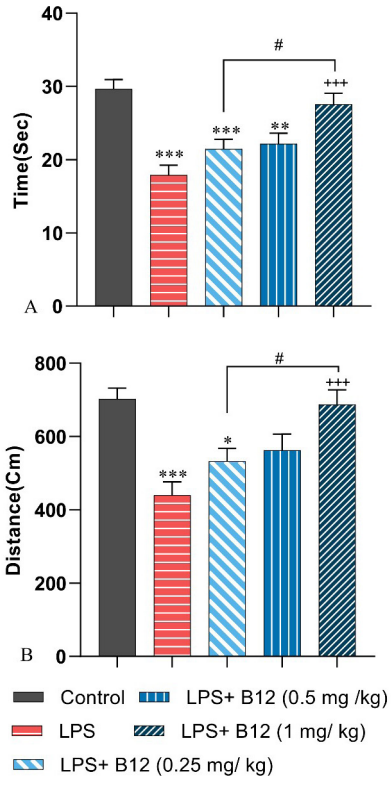


**Figure 2.** The time spent (A) and distance traveled (B) in the target quadrant during the probe trial of the Morris water maze in rat groups
Data are presented as mean±SEM (n=10 per group). Statistical significance: *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs control group; +++$P$<0.001 vs LPS group; #$P$<0.05 for comparisons between treatment groups.
SEM: Standard error of the mean; LPS: Lipopolysaccharide

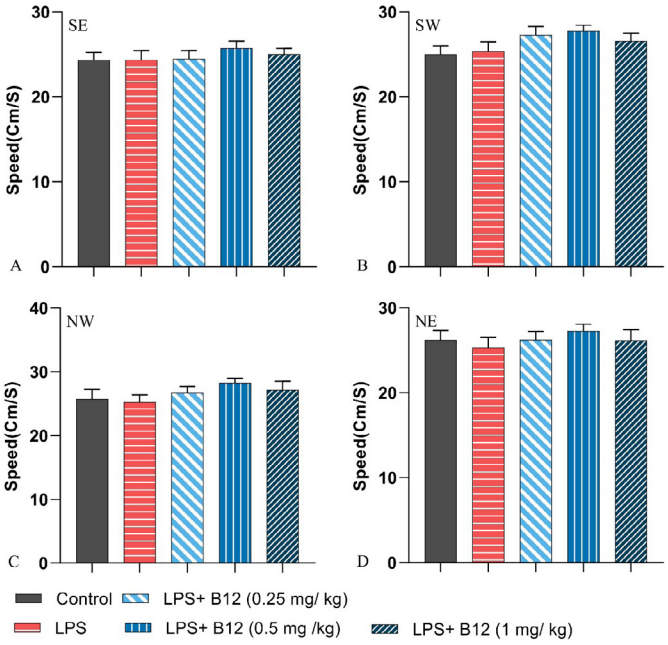


**Figure 3.**The traveling speed of rats in each quarter
Data are presented as mean±SEM (n=10 per group).
SEM: Standard error of the mean; LPS: Lipopolysaccharide

between LPS + B12 (1 mg/kg) and LPS + B12 (0.25 mg/kg) ($P$<0.05). The results also showed (Figure 3) that there was no significant difference among the groups in the speed in the target area (SE) and non-target areas (SW, NW, and NE).

***Passive avoidance test results***

After habituation, animals received a shock in the dark compartment, and then the test was conducted 3, 24, 48, and 72 hr after the shock. Analysis by the Kruskal–Wallis test showed that there was a significant difference in latency time to enter the dark chamber at 3 hr (H (4)=29.83; $P$<0.001) after delivery of a shock. The results of analysis by Dunn's pairwise test In the 3-h post-delivery shock showed that (Figure 4A), the LPS group exhibited significantly reduced latency ($P$<0.001), and every dose of the treatment was able to increase the latency time compared to the LPS ($P$<0.05 for 0.25 mg/kg, $P$<0.01 for 0.5 mg/kg, and $P$<0.001 for 1 mg/kg doses). In the subsequent test, which was conducted the next day, 24 hr after the shock (Figure 4B), there was a significant difference in latency time to enter the dark chamber (H (4) = 35.64; $P$<0.001). Further analysis showed that the difference between control and LPS remained statistically significant ($P$<0.001). The medium (0.5 mg/kg) and highest (1 mg/kg) doses of B12 resulted in statistically significant improvement in latency compared to LPS ($P$<0.001). In contrast, the low dose was the only dose that had no significant difference from LPS, and it was also markedly lower in this factor compared to the control ($P$<0.05). Both the medium dose and the high dose of B12 were significantly higher than the low dose as well ($P$<0.01). This pattern continued in the 48- and 72-hour tests (Figures 4C and 4D); the results showed that there was a significant difference among the groups at 48 hr (H (4)= 32.30; $P$<0.001) and 72 hr post-delivery shock times (H (4)=29.91; $P$<0.001). Further analysis showed that the latency in LPS groups was shorter than that in the control group at 48 hr ($P$<0.001) and 72 hr ($P$<0.001) post-delivery shock times. The difference between the low dose of B12 and the LPS was not significant, and the medium and high doses of B12 had longer latencies than the LPS group ($P$<0.05 - $P$<0.001).

The amount of time spent in the light compartment of the PA apparatus was also recorded over 72 hr (Figures

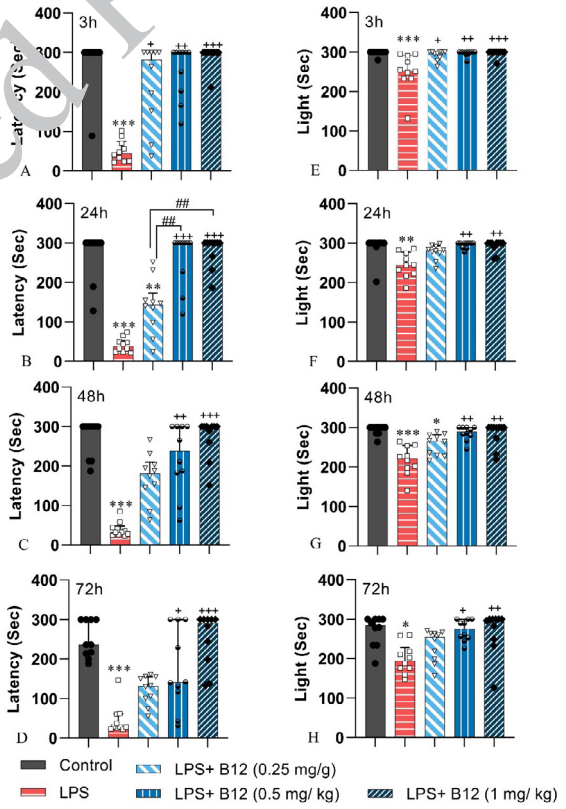


**Figure 4.** Latency to enter the dark compartment (A) and time spent in the light compartment (B) in the passive avoidance test in rat groups
Data are presented as median with interquartile range (n=10 per group). Statistical significance: *$P$<0.05, **$P$<0.01, and ***$P$<0.001 vs control group; +$P$<0.05, ++$P$<0.01, +++$P$<0.001 vs LPS group
LPS: Lipopolysaccharide

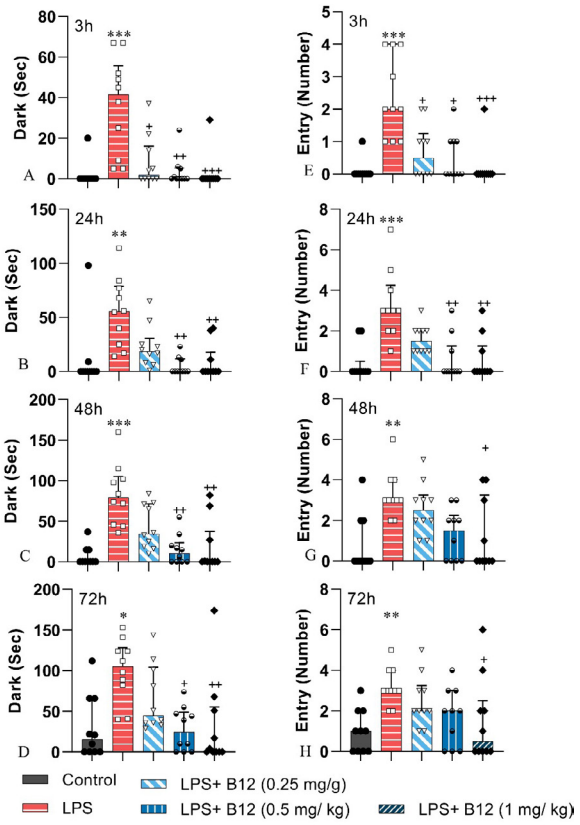


**Figure 5.** Time spent in the dark compartment (A) and number of entries into the dark compartment (B) in the passive avoidance test in rat groups. Data are presented as median with interquartile range (n=10 per group). Statistical significance: *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs control group; +$P$<0.05, ++$P$<0.01, +++$P$<0.001 vs LPS group.
LPS: lipopolysaccharide

4E-4H). The light time showed a significant difference among the groups at 3(H (4)=25.80; $P$<0.001, 24 (H (4) =26.43; $P$<0.001), 48 (H (4)=27.44; $P$<0.01), and 72 hr (H (4)=17.96; $P$<0.01) post-shock times. The gap between control and LPS remains significant throughout the period ($P$<0.001 for 3, $P$<0.01 for 24, $P$<0.001 for 48, and $P$<0.05 for 72 hr). The low dose of treatment was less successful compared to the medium and high doses. There was a significant difference between the 0.25 mg dose and the two other doses, and it was effective in increasing the light time only at 3 hr ($P$<0.05; Figure 4G) but not at 24 (Figure 4E), 48 (Figure 4F), and 72 hr (Figure 4H). After 3($P$<0.01 and $P$<0.001 for 0.5 and 1 mg/kg doses respectively), 24 ($P$<0.01 for both doses), 48 ($P$<0.01 for both doses), and 72 hr ($P$<0.05 and $P$<0.01 for 0.5 and 1 mg/kg doses respectively), the medium and highest doses were effective in increasing the light time.

In addition to latency and light time, dark time and number of entries to the dark compartment were recorded to further analyze the animal's memory of the shock. The dark time was significantly different between the groups at all 3(H (4)=25.40; $P$<0.001), 24(H (4)=26.43; $P$<0.001), 48(H (4)=27.44; $P$<0.001), and 72 hr (H (4)=17.96; $P$<0.01) times after shock delivery. The LPS-injected group had longer dark times than the control group at all 3 ($P$<0.001), 24 ($P$<0.01), 48 ($P$<0.001), and 72 hr ($P$<0.05) after the shock (Figures 5A, 5B, 5C, and 5D). The lowest dose (0.25 mg/kg) decreased the dark time at 3 ($P$<0.05), but both 0.5 mg/kg and 1 mg/kg doses were able to decrease the dark time at all 3 ($P$<0.01 and $P$<0.001, respectively), 24 ($P$<0.01 for both doses), 48 ($P$<0.01 for both doses), and 72 hr ($P$<0.05 and $P$<0.01, respectively) (Figures 5A, 5B, 5C, and 5D).

The difference was also reflected in the number of entries between the groups at all 33(H (4)=25.52; $P$<0.001), 24(H (4)=25.75; $P$<0.001), 48(H (4)=16.94; $P$<0.01), and 72 hr (H (4)=14.72; $P$<0.01) times after the shock. The difference between the LPS and control groups was revealed (Figures 5E-5H), with the LPS group averaging a higher number of entries into the dark compared to the control ($P$<0.001 for 3 and 24 hours, $P$<0.01 for 48 and 72 hr). The treatments at all doses were able to decrease the number of entries to a considerable degree at 3 hr ($P$<0.05 for 0.25 and 0.5 mg/kg doses and $P$<0.001 for the 1 mg/kg dose). The medium and highest doses ($P$<0.01), but not the lowest dose, were able to decrease the dark entries at 24 hr. At 48 and 72 hr, only the highest 1 mg/kg dose ($P$<0.05) was able to decrease the number of dark entries at 72 hr, but the lowest and the medium doses were not effective (Figures 5E, 5F, 5G, and 5H).

### *AChE activity*

A significant difference was seen between the groups in hippocampal (F(4, 40)=9.56, $P$<0.001) and cortical (F(4, 45) = 8.97, $P$<0.001) AChE activity. LPS was able to considerably increase AChE activity in both the hippocampus and cortex ($P$<0.001 for both). A 0.25 mg dose of vitamin B12 was able to decrease AChE activity in the cortex ($P$<0.01) (Figure 6B) but not in the hippocampus (Figure 6A). The AChE activity in the low-dose treatment was still significantly higher than the control in the hippocampus ($P$<0.05). Both 0.5 and 1 mg/kg doses were able to considerably attenuate the activity in both the hippocampus ($P$<0.01 and $P$<0.001 for medium and highest doses, respectively) and cortex ($P$<0.001 for both medium and highest doses) compared to the LPS group (Figures 6A and 6B).

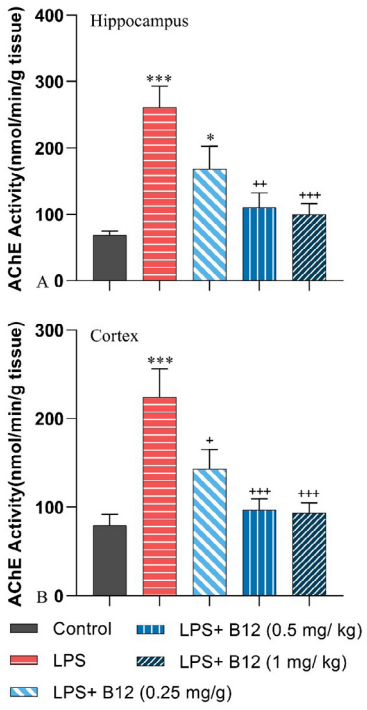


**Figure 6.** AChE activity in the hippocampus (A) and cortex(B) in rat groups
Data are presented as mean±SEM (n=9-10 per group). Statistical significance: *$P$<0.05, ***$P$<0.001 vs control group; +$P$<0.05, ++$P$<0.01, +++$P$<0.001 vs LPS group
AChE: Acetylcholinesterase; SEM: Standard error of the mean; LPS: Lipopolysaccharide

### *Oxidative stress results*

A significant difference was seen between the groups in the hippocampal MDA (F(4, 45)=16.93, *P*<0.001). MDA, as an oxidative stress marker, was significantly increased in the LPS group compared to the control (*P*<0.001) (Figure 7A). The low dose was unable to meaningfully alleviate MDA levels and remained at a significantly higher level than the control (*P*<0.001), but the subsequent higher doses were able to reduce this marker (*P*<0.01 for the 0.5 mg/kg dose, *P*<0.001 for the 1 mg/kg dose).

Total thiol content (F(4, 45)=13.50, *P*<0.001) and SOD activity (F(4, 45)=09.93, *P*<0.001) were also significantly different between the groups. Total thiol content and SOD, two anti-oxidants, were both considerably depleted as a result of LPS injection (Figures 7B and 7C; *P*<0.001 for both SOD and total thiol content). Thiol content (Figure 7B) was not significantly affected by the low and medium doses of the treatment and remained lower than the control group, and its levels were comparable to LPS (*P*<0.001 relative to control). The highest dose, on the other hand, was able to restore thiol content compared to the low and medium doses of B12 and also the LPS group (*P*<0.01 compared to LPS and *P*<0.05 compared to low and medium doses of B12). Unlike the thiol content, the low and medium doses of B12 were able to increase SOD relative to LPS (*P*<0.05), but the biggest effect was still observed in the high dose of our treatment (*P*<0.001).

### *Inflammatory cytokines*

A significant difference was seen between the groups in TNF-α (F(4, 19)=04.71, *P*<0.01). TNF-α mRNA expression was increased as a result of LPS (Figure 8A) and was elevated considerably in relation to the control (*P*<0.05). This factor was also markedly reduced as a result of treatment with B12, with every dose successfully reducing this marker (*P*<0.05 for 0.25 and 1 mg/kg, *P*<0.01 for 0.5 mg/kg). Other inflammatory markers such as IL-1β (F(4, 20)=06.89, *P*<0.01) and IL-6 (F(4, 20)=07.34, *P*<0.01) were also significantly different among the groups. Other inflammatory indicators, including IL-1β and IL-6, were also elevated as a result of LPS (*P*<0.01 for both IL-1β and IL-6) (Figures 8B and 8C). As for IL-1β (Figure 8B), every dose of B12 was able to decrease the expression of IL-1β significantly compared to LPS (*P*<0.01 for every dose). B12 was also able to decrease the expression of IL-6 (Figure 8C); the low, medium, and highest doses reduced the expression significantly (*P*<0.01 for low and medium doses and *P*<0.05 for the high dose).

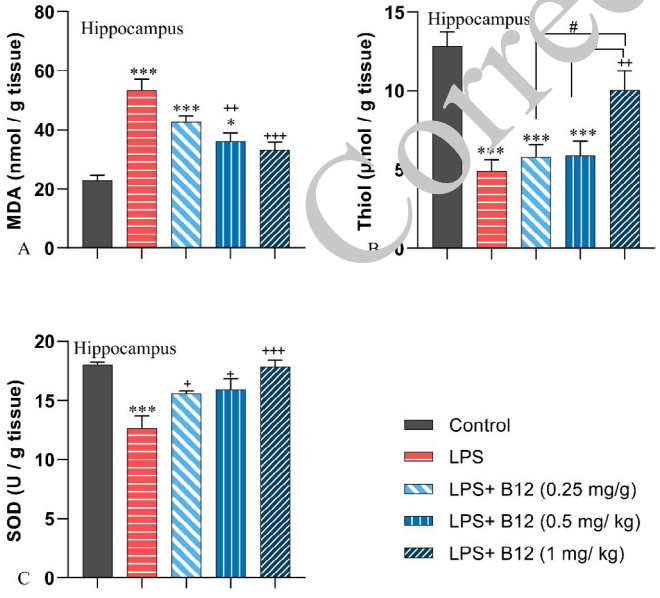


**Figure 7.** MDA (A), total thiol (B), and SOD (C) levels in the hippocampus in rat groups

Data are presented as mean±SEM (n=10 per group). Statistical significance: **P*<0.05, ****P*<0.001 vs control group; +*P*<0.05, ++*P*<0.01, +++*P*<0.001 vs LPS group; #*P*<0.05 for comparisons between treatment groups

MDA: Malondialdehyde; SOD: Superoxide dismutase; SEM: Standard error of the mean; LPS: Lipopolysaccharide

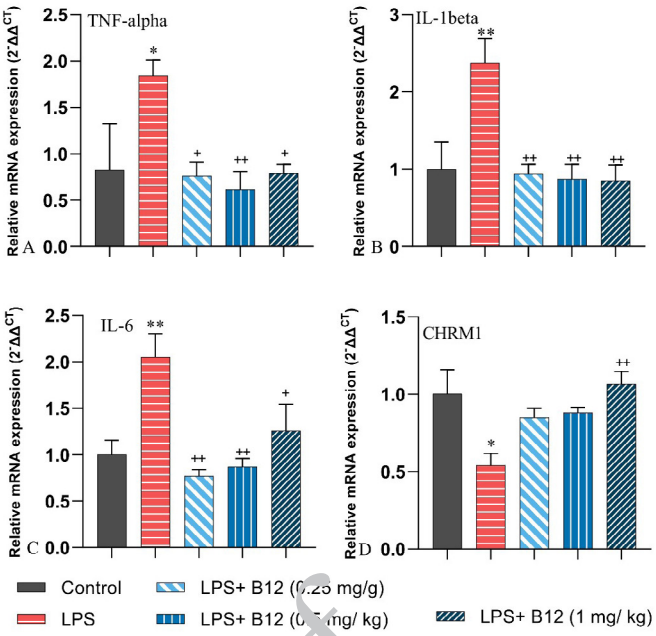


**Figure 8.** Relative mRNA expression (2^−ΔΔCT) of TNF-α (A), IL-1β (B), IL-6 (C), and CHRM1 (D) in the hippocampus in rat groups

Data are presented as mean±SEM (n=4-5 per group). Statistical significance: **P*<0.05, ***P*<0.01 vs control group; *P*<0.05, ++*P*<0.01 vs LPS group.

TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; CHRM: Muscarinic acetylcholine receptor; SEM: Standard error of the mean; LPS: Lipopolysaccharide

### *Muscarinic receptor*

A significant difference was revealed between the groups in CHRM1 (F(4, 20)=04.96, *P*<0.01). CHRM1 expression, as a marker of cholinergic function, was also decreased as a result of LPS injection (*P*<0.05) (Figure 8D), and although the low and medium doses of B12 were able to increase the average amount of CHRM1 expression, the difference between these two doses and LPS was not statistically significant. On the other hand, the highest dose of B12, 1 mg/kg, was able to increase the expression of this receptor considerably (*P*<0.01).

## Discussion

The assay of behavioral tests and biochemical and PCR experiments showed that impaired performance of rats in both MWM and passive avoidance tests after LPS injection was accompanied by increased levels of mRNA expression of inflammatory cytokines, increased concentration of MDA, a decrease in thiol and SOD, an increase in AChE activity, and a decrease in mRNA levels of CHRM1. In the present study, vitamin B12 treatment markedly attenuated the

LPS-induced behavioral, biochemical, and gene expression changes, suggesting a potential neuroprotective effect against inflammation-associated cognitive impairment. Neuroinflammation is a major contributing factor to many neurological disorders and has been heavily implicated in recent years as a major part of the pathophysiology of neurodegenerative diseases such as AD (27). In the current research, LPS injection produced a neuroinflammatory state, which was confirmed by increased levels of mRNA expression of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in the hippocampus. Neuroinflammation induced by LPS has been frequently shown to be associated with cognitive impairments (7, 28, 29). In the current study, the rats in the LPS-injected group had a longer time and distance to reach the platform during 5 days of MWM training. The rats in the LPS group also spent a shorter time and traveled a shorter distance in the target area in the probe test of MWM. In addition, the rats in the LPS group had a shorter latency to enter the dark, and they spent a longer time in the dark and entered the dark compartment more frequently. Our study demonstrated that systemic administration of LPS can produce significant memory impairment and neuroinflammation, and LPS injection is a reliable model for neuroinflammation since LPS injection significantly increased the expression of inflammatory cytokines such as IL-1β, TNF-α, and IL-6, as was previously shown (1).

The cholinergic system was also modulated in our findings; AChE activity was substantially increased with administration of LPS, and expression of CHRM1 was reduced, further confirming previous findings that suggested pro-inflammatory cytokines and chronic neuroinflammation lead to cholinergic dysfunction (30, 31). It has also been previously reported that the inflammatory cytokines produced during neuroinflammation lead to beta-amyloid (Aβ) production (32).

The neuroprotective, anti-inflammatory, and anti-oxidant effects of B12 have been previously reported (15, 33). In the current study, our treatment groups spent less time and traveled shorter distances to find the platform in the first five days of MWM, which suggests a more functional memory and learning process in our treatment groups. After the 5 days of MWM, in the probe test, the B12-administered groups spent more time and traveled a longer distance in the target quadrant, where the escape platform was placed in the past five days. It is important to note that the average speed in each quarter of the MWM test was also measured, and there was no significant difference between groups; therefore, it seems that the difference in performance in the MWM test was due to memory and learning, but not a difference in motor performance. Similar effects were found in the PA test as well. The latency was increased as a result of administration of B12, with the medium and the highest doses exhibiting a better protective effect against LPS than the lowest doses. In addition, B12, especially at medium and highest doses, decreased the dark time and entries while increasing the light time in the PA test.

The vitamin B family and B12 have been extensively reported to have a role in memory and learning, and deficiency of these nutrients can impair memory (34) as well as short-term memory loss (35). Some studies have found a significant association between B12 intake and risk of dementia and AD in the elderly (36), and there have been reports of animal studies that show B12 can be used to treat memory dysfunction in aged rats with tau pathologies (37).

The present results also showed that all doses of B12 attenuated the expression of inflammatory cytokines in the hippocampus. Levels of B12 in serum have been negatively correlated with inflammatory cytokines (33). This is consistent with previous reports on the anti-inflammatory properties of B12 (38, 39). Vitamin B12 and folic acid together also have ameliorating effects on inflammatory markers (40), and high doses of B12 have been reported to be effective in the treatment of septic shock in the clinical setting (41); supplementing donepezil with B12 can also lower inflammation (42). The observed reduction in TNF-α, IL-1β, and IL-6 mRNA expression following vitamin B12 treatment suggests that modulation of these inflammatory pathways may contribute to its neuroprotective effects. However, inflammatory indicators at the protein level and more precise methods need to be investigated.

Previous studies have shown that LPS activates the toll-like receptor (TLR)4 signaling pathway, which subsequently stimulates nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), and NLRP3 inflammasome signaling, leading to increased production of pro-inflammatory cytokines (43, 44). While the exact signaling pathways were not directly examined in the present study, it has been previously reported that vitamin B12 and B-complex were able to reduce the inflammatory mediators secreted by LPS-stimulated microglia and change the microglia toward an M2-type phenotype profile, thereby attenuating neuronal toxicity(45). Therefore, some histopathological analyses and the assessment of glial activation markers, such as Iba-1 and glial fibrillary acidic protein (GFAP), are suggested to be done in further studies. In addition, the beneficial effects of vitamin B12 on brain ischemia are suggested to be mediated by TLR-4/NF-κB / MAPK signaling pathway (56), which may have a role in the results seen in the present study; however, it needs to be further investigated.

Administration of B12 also successfully improved the expression of CHRM1 and decreased the activity of AChE in the present study. A previous study has reported a dose-dependent effect of B12 on the rat model of cholinergic dysfunction and has shown that the high dose of B12 has a positive effect on the cholinergic system (46), which aligns with our results. In the present study, the highest dose of vitamin B12 significantly increased CHRM1 expression, while the medium and high doses significantly reduced AChE activity. Although the previous study was conducted in an AD model, our findings extend this evidence by demonstrating that vitamin B12 also modulates cholinergic signaling in an LPS-induced neuroinflammation model.

Given the complex and significant impact of the cholinergic system on memory (58, 59), some of the improvements observed in the treatment groups in the present study may reflect effects on cholinergic neurons.

Oxidative stress also plays a major role in AD (47) and happens before the accumulation of Aβ (48) and can be a link between tau pathology and Aβ plaque formation (49). B12 was able to decrease MDA and also prevented the reduction of SOD and thiol content. B vitamins and B12 are involved in several functions and pathways within the nervous system (50) and have been linked to neurodegenerative disorders as well (51). In the present study, the effects of vitamin B12 for prevention and mitigation of memory impairment by LPS might at least in part be due to anti-oxidant functions

of B12 (52, 53). It was found in the present study that B12 administration improved thiol content and SOD activity and decreased MDA concentration in the hippocampus. Collectively, it was shown here in the present study that B12 improved learning, memory, and cholinergic function, and attenuated neuroinflammation and oxidative stress. In the current research, B12 alone was not examined in the control rats; it would be better to evaluate the effects of B12 in control conditions. In some previous studies, the beneficial effects of vitamin B12 are predominantly observed under pathological but non-control conditions. In a paraquat-induced neurotoxicity model, a vitamin B12-only group was included in the experimental design, and no significant difference between the vitamin B12-only and control groups was reported, whereas the protective effects of vitamin B12 were evident under paraquat-induced oxidative stress (54). Likewise, in a rat model of sciatic nerve crush injury, vitamin B12 enhanced functional recovery and nerve regeneration after injury, further supporting the view that its neuroprotective and regenerative effects are primarily observed under pathological conditions (68). Therefore, given that previous studies have primarily demonstrated the beneficial effects of vitamin B12 under pathological conditions, the absence of a vitamin B12-only group is unlikely to substantially affect the interpretation of the neuroprotective effects observed in the LPS model in the current study. Nevertheless, future studies should include such a group to directly assess the independent effects of vitamin B12 under physiological conditions.

## Conclusion

Our work offers evidence for the benefits of B12 administration to mitigate cognitive impairment and memory disruption caused by LPS and inflammation. These findings were confirmed with an assay of behavioral tests for assessing the memory of animals and markers for oxidative stress and inflammation, as well as AChE and CHRM1, to also look into the possible role of B12 in regulating the cholinergic system in the brain. In our tests, the most pronounced effects on memory and learning were observed at the highest treatment dose, but the low and medium doses also showed positive effects. The current research also suggests that vitamin B12 might have positive effects on memory due to its effects on the cholinergic system and its inhibitory effects on AChE, as well as by considerably increasing the expression of acetylcholine receptors in the hippocampus. Our work suggests a correlation between inflammation, cholinergic activity, and memory, and treatment with B12 was able to improve these factors. This study provides evidence for the use of vitamin B12 for treatment and prevention of memory impairment, mitigating neuroinflammation and oxidative damage, although more preclinical and translational work is warranted.

### *Limitation*

Despite these promising results, the current work has several limitations that should be acknowledged. First, this study does not provide exact mechanisms for the effects of vitamin B12, and it would be beneficial to further study the underlying mechanisms and pathways through which B12 exerts its effects and how it improves memory and learning. Second, a control group that only received vitamin B12 was not included; therefore, the independent effects of vitamin B12 on cognitive function, oxidative stress, inflammatory responses, and cholinergic signaling could not be distinguished from its protective effects against LPS-induced alterations. Third, inflammatory mediators were evaluated only at the mRNA level, and no protein-level validation (e.g., ELISA or Western blotting) was performed. In addition, histopathological analyses and the assessment of glial activation markers, such as Iba-1 and GFAP, were not conducted. Although our findings suggest that vitamin B12 may modulate neuroinflammatory responses, the precise molecular and cellular mechanisms underlying these effects remain to be elucidated. Finally, the long-term effects of vitamin B12 treatment were not investigated and should be addressed in future studies.

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## Authors' Contributions

M H, E T, and A R designed the experiments; Z KH, R S, H H, and S M conducted the behavioral experiments and collected the data; A H, R S, and Z KH conducted the biochemical experiments and collected the data; A R and M H discussed the results and strategy; M H supervised, directed, and managed the study; A R supervised the biochemical experiments; Z KH and S M prepared the initial version of the manuscript; S M, M H, and A R finalized the manuscript.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Declaration

We acknowledge that an AI tool (QuillBot) was used for language editing and grammar checks. No AI tool was used for the generation of the manuscript, graphs, or data analysis.

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