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ORIGINAL PAPER

The Effect of NeuroAid (MLC901) on Cholestasis-Induced Spatial Memory Impairment with Respect to the Expression of BAX, BCL-2, BAD, PGC-1α and TFAM Genes in the Hippocampus of Male Wistar Rats

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Abstract

Cholestasis is a bile flow reduction that is induced following Bile Duct Ligation (BDL). Cholestasis impairs memory and induces apoptosis. Apoptosis consists of two pathways: intrinsic and extrinsic. The intrinsic pathway is modulated by *BCL-2* (B cell lymphoma-2) family proteins. *BCL-2* (a pro-survival *BCL-2* protein) has anti-apoptotic effect, while *BAD* (*BCL-2*-associated death) and *BAX* (*BCL-2*-associated X), the other members of *BCL-2* family have pro-apoptotic effect. Furthermore, *TFAM* (mitochondrial transcriptional factor A) is involved in transcription and maintenance of mitochondrial DNA and *PGC-1α* (peroxisome proliferator-activated receptor γ coactivator-1α) is a master regulator of mitochondrial biogenesis. On the other hand, NeuroAid is a Traditional Chinese Medicine with neuroprotective and anti-apoptosis effects. In this study, we evaluated the effect of cholestasis on spatial memory and expression of *BCL-2*, *BAD*, *BAX*, *TFAM*, and *PGC-1α* in the hippocampus of rats. Additionally, we assessed the effect of NeuroAid on cholestasis-induced cognitive and genetic alterations. Cholestasis was induced by BDL surgery and NeuroAid was injected intraperitoneal at the dose of 0.4 mg/kg. Furthermore, spatial memory was evaluated using Morris Water Maze (MWM) apparatus. The results showed cholestasis impaired spatial memory, increased the expression of *BAD* and *BAX*, decreased the expression of *TFAM* and *PGC-1α*, and did not alter the expression of *BCL-2*. Also, NeuroAid decreased the expression of *BAD* and *BAX* and increased the expression of *TFAM*, *PGC-1α*, and *BCL-2*. In conclusion, cholestasis impaired spatial memory and increased the expression of pro-apoptotic genes. Also, cholestasis decreased the expression of *TFAM* and *PGC-1α*. Interestingly, NeuroAid restored the effects of cholestasis.

Keywords Cholestasis · Spatial memory · NeuroAid · Gene expression · Hippocampus · Apoptosis

Introduction

Bile formation is a special function of liver that is crucial for liver health [1]. Bile Duct Ligation (BDL) disrupts bile secretion and induces liver damage [2]. BDL also induces hepatic fibrosis, necrosis, and apoptosis [3, 4]. Furthermore, BDL induces cholestasis [2, 5]. Cholestasis reduces the bile flow and induces liver damage [6–8]. Cholestasis also impairs learning and memory in different cognitive tasks [9, 10]. It has been revealed that cholestasis disrupts memory retrieval in one-trial step-down memory task in mice [11]. Cholestasis also impairs spatial recognition memory in Y-maze task [12]. Furthermore, mild BDL can impair learning in Y-maze task in rats [13]. Interestingly, human studies have shown that biliary cirrhosis and liver diseases can induce cognitive impairments and dementia [14, 15]. It should be noted that molecular mechanisms of cholestasis involved in cognitive impairments are largely unknown. However, it has been suggested that BDL-induced hyperammonia may be a main factor for cognitive and neurological impairments [16, 17]. In addition, disruptions in neurotransmitter systems including glutamatergic, cholinergic, dopaminergic, opioidergic, GABAergic, and serotonergic have

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been observed following BDL or liver diseases [18–20]. Further, as mentioned before, BDL plays an important role in the induction of necrosis and apoptosis [21–23].

Apoptosis is a form of programmed cell death. The inappropriate form of apoptosis (too little or too much) induces neurodegenerative and autoimmune diseases, and various types of cancer [24]. Apoptosis occurs via two pathways: the extrinsic and the intrinsic [25]. The intrinsic pathway (also called mitochondria-dependent) is modulated by *BCL-2* (B cell lymphoma-2) family of proteins [26, 27]. *BCL-2* proteins integrate both death and survival signals, and based on the composition of typical BH domains are divided into anti-apoptotic and pro-apoptotic groups [28, 29]. The integrity of the Outer Mitochondrial Membrane (OMM) is regulated by anti-apoptotic *BCL-2* proteins including *BCL-2* (a pro-survival *BCL-2* protein) [30, 31]. Furthermore, pro-apoptotic *BCL-2* proteins such as *BAD* (*BCL-2*-associated death) and *BAX* (*BCL-2*-associated X) activate caspases and promote cell death via releasing mitochondrial Inter-Membrane Space (IMS) proteins [32, 33].

TFAM (mitochondrial transcriptional factor A) has a critical role in transcription and maintenance of mitochondrial DNA (mtDNA) [34, 35]. *TFAM* also protects mtDNA from free radicals [36]. Abnormal function of mtDNA can lead to severe disorders. For example, decrease in functional mtDNA abundance may induce Parkinson's disease, Alzheimer's disease (AD), and Amyotrophic lateral sclerosis (ALS) [37, 38]. On the other hand, *PGC-1α* (peroxisome proliferator-activated receptor γ coactivator-1α) is a master regulator of mitochondrial biogenesis and regulates energy metabolism and antioxidant pathways [39, 40]. *PGC-1α* has a critical role in the pathogenesis of various diseases such as AD [41].

The hippocampus has high expression of *BCL-2* proteins family including *BAX* and *BAD* [42]. It has been revealed that stress upregulates the expression of *BAX* and *BAD* [42]. Stress damages the neurons probably via induction of pro-apoptotic factors, while upregulation of anti-apoptotic *BCL-2* proteins may be a compensatory response [43, 44]. Furthermore, the hippocampus has high expression of *PGC-1α* and *TFAM* [40]. The expression of *PGC-1α* and *TFAM* is downregulated following dementia and AD in memory-related brain regions especially hippocampus [45].

NeuroAid (as a Traditional Chinese Medicine) has anti-apoptotic and neuroprotective effects [46, 47]. NeuroAid (MLC901) has nine herbal components per each capsule including 0.80 g Radix astragali, 0.16 g Radix salvia miltiorrhizae, 0.16 g Radix paeoniae rubra, 0.16 g Rhizoma chuanxiong, 0.16 g Radix angelicae sinensis, 0.16 g Car-thamus tinctorius, 0.16 g Prunus persica, 0.16 g Radix polygalae, and 0.16 g Rhizoma acori tatarinowii [48]. NeuroAid inhibits necrosis and apoptosis of neurons in ischemic rats [49]. Also, NeuroAid plays an important role in the repair of neurovascular unit after ischemic stroke [50]. Recent pharmacological studies have shown that NeuroAid protects the brain from ischemic injury, decreases functional deficits in animal models of stroke, and prevents neural death in in-vitro models of excitotoxicity [51]. NeuroAid also induces synaptogenesis, promotes cell proliferation, and stimulates the development of dense axonal and dendritic networks [51]. In addition, BDNF (brain-derived neurotrophic factor) and VEGF (vascular endothelial growth factor), the key mediators of adaptive remodeling of surviving neurons and neural networks [52] are upregulated following NeuroAid treatment [51, 53].

According to the mentioned findings, the goal of this research is to investigate the effect of NeuroAid on cholestasis-induced spatial memory impairment with respect to the expression of *BAX*, *BCL-2*, *BAD*, *PGC-1α*, and *TFAM* in the hippocampus of male Wistar rats.

Material and Method

Animals

In the present study, male Wistar rats (220-240 g, 11–12 weeks old) bred at Iranian National Center for Addiction Studies (INCAS), Tehran, Iran were used. Rats were kept in the lab with a 12/12 h light-dark cycle and standard temperature (22 ± 2 °C). Rats were placed in Plexiglas cages in groups of 4 and free access to food and water was provided except during the experiments. Each experimental group consisted of eight rats. Furthermore, the behavioral experiments were done only during the light phase. All the experiments were done in accordance with the guidelines of NIH (NIH Guide for the Care and Use of Laboratory Animals) and the guidelines of Institute for Cognitive Science Studies (ICSS), Tehran, Iran.

Bile Duct Ligation (BDL) Surgery

Rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (50 mg/kg) plus xylazine (5 mg/kg). During surgery, the common bile duct was located and ligated using 4 − 0 silk at two points anterior to the pancreas and posterior to the hilum of the liver. One ligation was made just above the duodenum; the second ligation was made approximately 2 mm above the first ligation and then transected at the midpoint between the two ligatures [11, 54]. Sham-ligation surgery was performed by locating and manipulating the common bile duct. Sterile 0.9% NaCl solution (1 mL/rat) was injected intraperitoneal immediately after the surgery. All surgeries were performed using aseptic technique. After the operation, each rat was placed in a

cage by itself to prevent wound dehiscence and 4 h later was transferred to its original cage [11, 55].

Drug

NeuroAid (MLC901) (Moleac, Singapore) including 9 herbal components per each capsule: 0.80g Radix astragali, 0.16g Radix salvia miltiorrhizae, 0.16g Radix paeoniae rubra, 0.16g Rhizoma chuanxiong, 0.16g Radix angelicae sinensis, 0.16g Car-thamus tinctorius, 0.16g Prunus persica, 0.16 g Radix polygalae, and 0.16g Rhizoma acori tatarinowii [48] was injected intraperitoneal at the dose of 0.4 mg/kg.

Experimental Groups

This study consisted of four experimental groups including: Sham of BDL with saline (1 mL/kg), Sham of BDL with NeuroAid (0.4 mg/kg), BDL with saline (1 mL/kg), and BDL with NeuroAid (0.4 mg/kg). NeuroAid (Moleac, Singapore) was diluted in saline (used as a vehicle) at concentration of 0.4 mg/mL (stock solution) and incubated under agitation for 1 h at 37 °C. The solution was strained using a 0.22-µm filter. One day after BDL surgery, NeuroAid (0.4 mg/kg) was injected intraperitoneal; and then, was injected every other day up to 28 days [46]. After 28 days, spatial memory was assessed. 28-day period was selected because BDL induces a strong fibrotic response after 21 to 28 days [56]. Additionally, many studies have used a 28-day period to evaluate the effect of BDL on cognitive and non-cognitive functions [10, 57–59]. We designed sham of BDL with saline group to assess the effect of both injection stress and surgery stress on memory performance.

Morris Water Maze

Morris Water Maze (MWM) is a valid test to assess spatial learning and memory [60, 61]. MWM is a circular black tank (150 cm in diameter and 60 cm depth) filled with water to a depth of 30 cm. The water temperature in the tank was about 22° C. The visual signs were placed on the walls around the tank. The maze was divided into four quadrants and each quadrant had a starting location: north (N), south (S), west (W), and east (E). The hidden platform (10 cm in diameter) was submerged 1 cm beneath the surface of the water in the center of the target quadrant (the north-west quadrant) [62]. All rats had to find the hidden platform by referring to the visual cues. The test began after a 4-week treatment. One day before the start of learning trials, the rats were familiarized with the apparatus. The learning session consisted of eight trials with four different starting positions. The hidden platform was in the north-west quadrant (the target quadrant). A camera was located above the tank and attached to a computer. The performance of each rat was recorded by a smart video tracking software (BorjSanatAzma). During learning trials, the escape latency, the traveled distance, and the mean velocity of each rat to find the hidden platform were measured. After finding the platform, each rat was allowed to remain on the platform for 20s (to memorize visual cues). In learning trials, more time spent or longer distance traveled means poor spatial learning. 24h after learning trials, the probe test was performed. In the probe test rats were placed in the tank for 1 min, while the hidden platform was removed. The escape latency, the traveled distance, and the mean velocity of the rats only in the target quadrant were measured. In probe trial, more time spent or longer distance traveled in the target quadrant means better spatial memory. After the probe test, we performed visible test with the visible platform to ensure that motor functions, visuo–motor abilities, or motivation of the rats to escape water or anything else did not influence the results.

Statistical Analysis for Spatial Memory

Statistical analyses were performed using SPSS software (V. 26). Given the normality of distribution and the homogeneity of data variance, the results were statistically evaluated using two-way ANOVA. Further analyses for individual "between-group" comparisons were done using post hoc Tukey test. In all comparisons, $P < 0.05$ was considered as statistical significance.

Hippocampus Sample Preparation

After performing the tests, rats were placed in a special chamber and killed with CO2 gas. The rat's brain was removed. After a brief wash with normal saline, the brain was placed inside a coronal section matrix suitable for the brain of rat weighing 200 to 250 g; and then, the hippocampus was separated and placed into the nitrogen tank. (Note: all stages of tissue extraction should not last longer than 2 min). After 24 h, fifty milligrams of hippocampus samples were diluted in 10 ng phosphate-buffered saline (PBS) (PH = 7.4) and homogenized by a homogenizer. The samples were centrifuged at 2000–3000 RPM for 20 min and the supernatants were collected carefully for measuring the expression level of *BCL-2*, *BAX*, *BAD*, *PGC-1α*, and *TFAM* genes. All samples were kept at −80 °C [63, 64].

Real-time PCR

In order to measure *BCL-2*, *BAX*, *BAD*, *PGC-1α*, and *TFAM* genes' mRNA expression, complementary DNA (cDNA) was prepared from the whole cellular RNA. The total RNA was extracted using BioFACT™ Total RNA

Table 1 Characteristics of the primers used in the real-time PCR assay

Localization	*Size (bp)*	*Sequence (5' – 3')*	*Primer*
F: 22	121	F: AAGTTCAACGGCACAGTCAAGG	GAPDH
R: 22		R: CATACTCAGCACCAGCATCACC	
F: 17	93	F: AGGGTGGCTGGGAAGGC	BAX
R: 17		R: TGAGCGAGGCGGTGAGG	
F: 24	134	F: ATCGCTCTGTGGATGACTGAGTAC	BCL-2
R: 24		R: AGAGACAGCCAGGAGAAATCAAAC	
F: 21	149	F: CCCGCGAACATATTCGAGAAG	PGC-1α
R: 20		R: TCGTTGTCAGTGGTCACGTC	
F: 24	128	F: AGAAAGCACAAATCAAGAGGAGAG	TFAM
R: 24		R: CAATTTCCCTTGAGGTGACTCATC	
F:20	126	F: GGAGCATCGTTCAGCAGCAG	BAD
R:23		R: CCATCCCTTCATCTTCCTCAGTC	

Prep Kit. The RNA was quantified by Picodrop Microliter Spectrometer. cDNA was prepared using BioFACT™ OneStep RT-PCR Kit according to the manufacturer's method in a final volume of 40 µl. Finally, the cDNA was stored at −20 °C [35].

In order to normalize target gene expression, GAPDH was used as the housekeeping gene. The primers used for the real-time PCR were *BCL-2*, *BAX*, *BAD*, *PGC-1α*, and *TFAM*.

After preparing hippocampus samples, the extracted RNA was purified and the high quality RNAs were selected and kept at − 80 °C until using for cDNA synthesis. Up to 1 µg RNA was converted to cDNA using Quantitect reverse transcription kit (Qiagen). The primers for Real-time PCR were designed and underwent an extensive search using BLAST tool. The characteristics of the primers used in this study have been summarized in Table 1. Real-time PCR was carried out using the following cycling conditions: 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Each complete amplification stage was followed by a dissociation stage: at 95 °C for 15 s, 60 °C for 30 s, then temperature was ramped up from 60 to 95 °C (at the rate of 0.03 °C/s). Melting curve analysis was performed according to the dissociation stage data and reactions.

Genetic Data Analysis

Quantitative analysis was performed by the measurement of Ct values during the exponential phase of amplification. Relative quantity of genes was determined using comparative Ct method and ΔCt was calculated as the difference between the Ct values of the target gene and the Ct value of GAPDH gene. The results were analyzed using this formula: Gene dosage ratio = $2^{-\Delta\Delta Ct}$. Statistical significance of difference was calculated using *t*-test.

Results

BDL Impaired Spatial Memory and NeuroAid Restored this Effect

Trials- The results of two-way ANOVA showed that the effect of BDL surgery, drug, and the interaction effect for traveled distance, escape latency, and mean velocity in both Sec. Introduction (the average data of trials 1 to 4) & 2 (the average data of trials 5 to 8) was not significant (Fig. 1). Thus, our data showed that spatial learning was not altered following BDL surgery or NeuroAid administration.

Probe- The results of two-way ANOVA for escape latency showed that the effect of BDL surgery ($F_{1, 28}$ = 4.32, $P < 0.05$), drug ($F_{1, 28}$ = 12.90, $P < 0.001$), and the

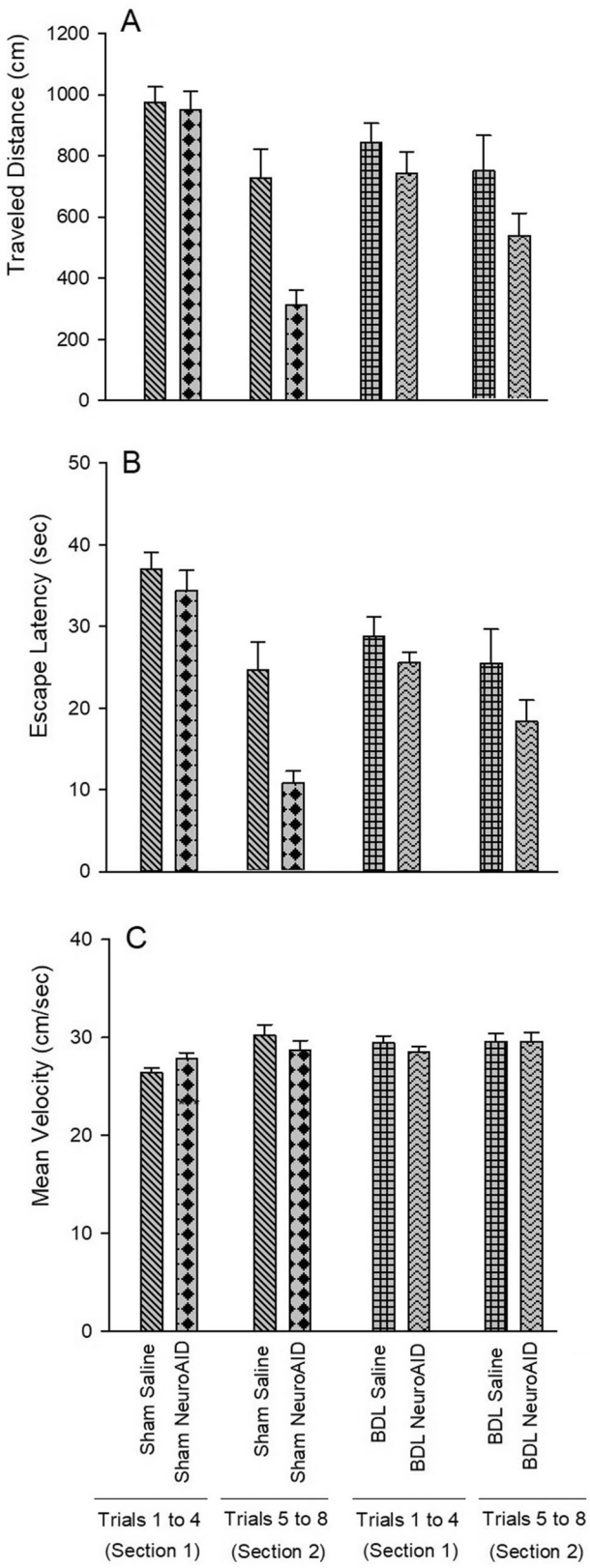

Fig. 1 Shows the effects of bile duct ligation surgery and NeuroAid ▶ injection on traveled distance (**a**), escape latency (**b**), and swimming speed (**c**) in the Morris Water Maze apparatus in two sections (Sec. Introduction: average data of trials 1 to 4, Sec. Material and Method: average data of trials 5 to 8), showing spatial learning performance. Two-way ANOVA and post hoc Tukey's were used to analyze data (n = 8)

interaction effect ($F_{1, 28} = 4.86$, $P < 0.05$) was significant. The results of two-way ANOVA for traveled distance showed that the effect of drug ($F_{1, 28} = 11.70$, $P < 0.01$), and the interaction effect ($F_{1, 28} = 8.90$, $P < 0.01$) was significant, while the effect of BDL surgery ($F_{1, 28} = 1.70$, $P > 0.05$) was not significant. The results of post hoc Tukey's showed that there is a significant difference between sham + saline and BDL + saline groups for traveled distance ($P < 0.001$) and escape latency ($P < 0.001$). The BDL rats could not distinguish the target quadrant. Therefore, the results showed that BDL decreased the traveled distance and the escape latency in the target quadrant, meaning impaired spatial memory consolidation. Also, post hoc Tukey's showed that there is a significant difference between BDL + saline and BDL-NeuroAid groups for traveled distance ($P < 0.001$) and escape latency ($P < 0.001$). Administration of NeuroAid in BDL group increased the traveled distance and the escape latency in the target quadrant, meaning that NeuroAid restored the impairment effect of cholestasis on spatial memory consolidation. Furthermore, the results of two-way ANOVA for the mean velocity were not significant (Fig. 2). Also, the results of the visible tests were not significant, meaning that motor functions, visuo–motor abilities, or motivation of the rats to escape water or anything else did not influence the results (data not shown).

Real Time PCR Analysis Shows Increased Expression of BAD and BAX in BDL Rats

BAX- The results of two-way ANOVA showed that the effect of BDL surgery ($F_{1, 28} = 149.75$, $P < 0.001$), drug ($F_{1, 28} = 231.25$, $P < 0.001$), and the interaction effect ($F_{1, 28} = 146.20$, $P < 0.001$) was significant. The results of post hoc Tukey's showed that the expression of *BAX* ($P < 0.001$) was increased in the BDL group, while NeuroAid reversed this effect ($P < 0.001$).

BAD- The results of two-way ANOVA showed that the effect of BDL surgery ($F_{1, 28} = 85.54$, $P < 0.001$), drug ($F_{1, 28} = 144.39$, $P < 0.001$), and the interaction effect ($F_{1, 28} = 86.19$, $P < 0.001$) was significant. The results of post hoc Tukey's showed that the expression of *BAD* ($P < 0.001$) was increased in the BDL group, while NeuroAid reversed this effect ($P < 0.001$).

BCL-2- The results of two-way ANOVA showed that the effect of BDL surgery ($F_{1, 28} = 752.24$, $P < 0.001$), drug ($F_{1, 28} = 898.33$, $P < 0.001$), and the interaction effect ($F_{1, 28}$

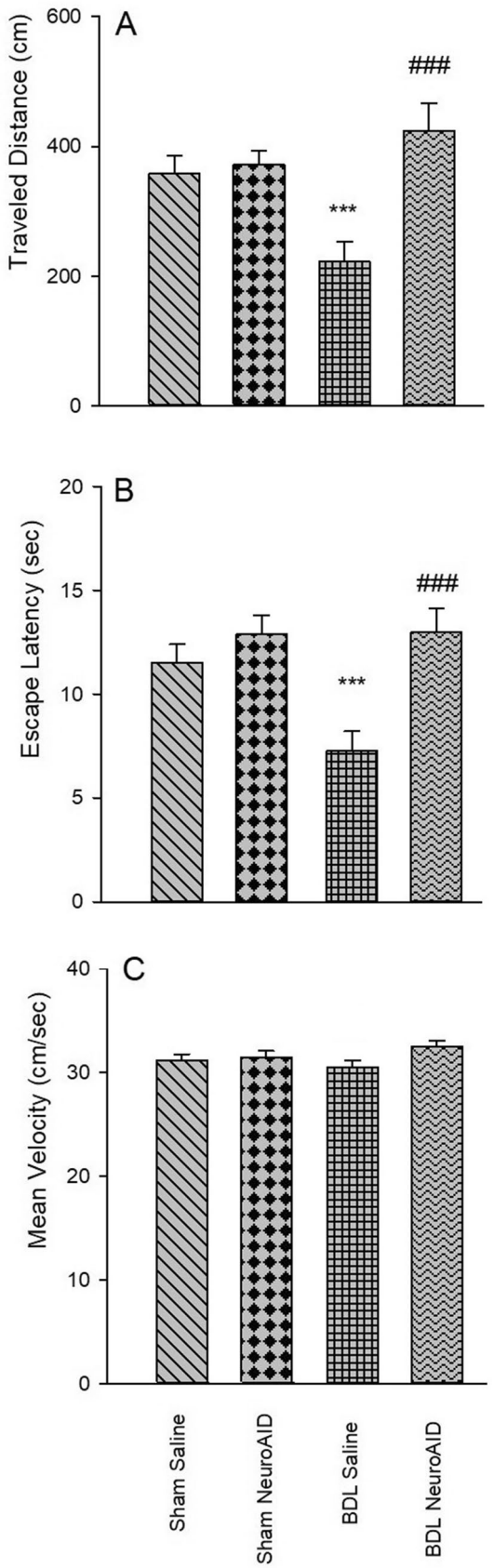

Fig. 2 Shows the effects of bile duct ligation surgery and NeuroAid injection on traveled distance (**a**), escape latency (**b**) and swimming speed (**c**) in the target quadrant of the Morris Water Maze apparatus, showing spatial memory consolidation. ***P < 0.001 different from sham + saline group, and ###P < 0.001 different from the BDL + saline group. Two-way ANOVA and post hoc Tukey's were used to analyze data (n = 8)

= 1139.92 $P < 0.001$) was significant. The results of post hoc Tukey's showed that the expression of *BCL-2* ($P < 0.001$) was increased in the BDL-NeuroAid group.

PGC-1α- The results of two-way ANOVA showed that the effect of BDL surgery ($F_{1, 28} = 633.65$, $P < 0.001$), drug ($F_{1, 28} = 782.39$, $P < 0.001$), and the interaction effect ($F_{1, 28} = 733.40$, $P < 0.001$) was significant. The results of post hoc Tukey's showed that the expression of *PGC-1α* ($P < 0.05$) was decreased in the BDL group, while NeuroAid reversed this effect ($P < 0.001$).

TFAM- The results of two-way ANOVA showed that the effect of BDL surgery ($F_{1, 28} = 3.73$, $P > 0.05$), and the interaction effect ($F_{1, 28} = 2.12$, $P > 0.05$) was not significant, while the effect of drug ($F_{1, 28} = 21.76$, $P < 0.001$) was significant. The results of post hoc Tukey's showed that the expression of *TFAM* ($P < 0.05$) was decreased in the BDL group, while NeuroAid reversed this effect ($P < 0.05$) (Fig. 3).

Discussion

Impairment Effect of BDL on Spatial Memory

Our results showed that spatial learning was not altered following BDL surgery or NeuroAid administration (Fig. 1 a and b). Furthermore, our data showed that BDL decreased the traveled distance and the escape latency in the target quadrant in the probe test meaning the impairment of spatial memory consolidation (Fig. 2a and b). Previous studies have shown the impairment effect of BDL on learning and memory. BDL impairs spatial performance in MWM apparatus and impairs learning in passive avoidance task [65]. It has been revealed that BDL impairs spatial memory consolidation in MWM apparatus [66]. Furthermore, BDL disrupts learning and memory retrieval in step-through passive avoidance task in rats [67]. BDL is a chronic liver injury that impairs learning and memory. It has been reported that acute or chronic liver injury impairs cognitive functions [68, 69]. Note that, the molecular mechanism of BDL involved in cognitive impairments is still unknown. In this research, BDL impaired spatial memory in MWM apparatus. It has been suggested that hyperammonia induced by the liver disease is one of the main factors responsible for cognitive impairments following BDL [16, 17]. Furthermore, disruptions in many neurotransmitter systems have been observed in liver

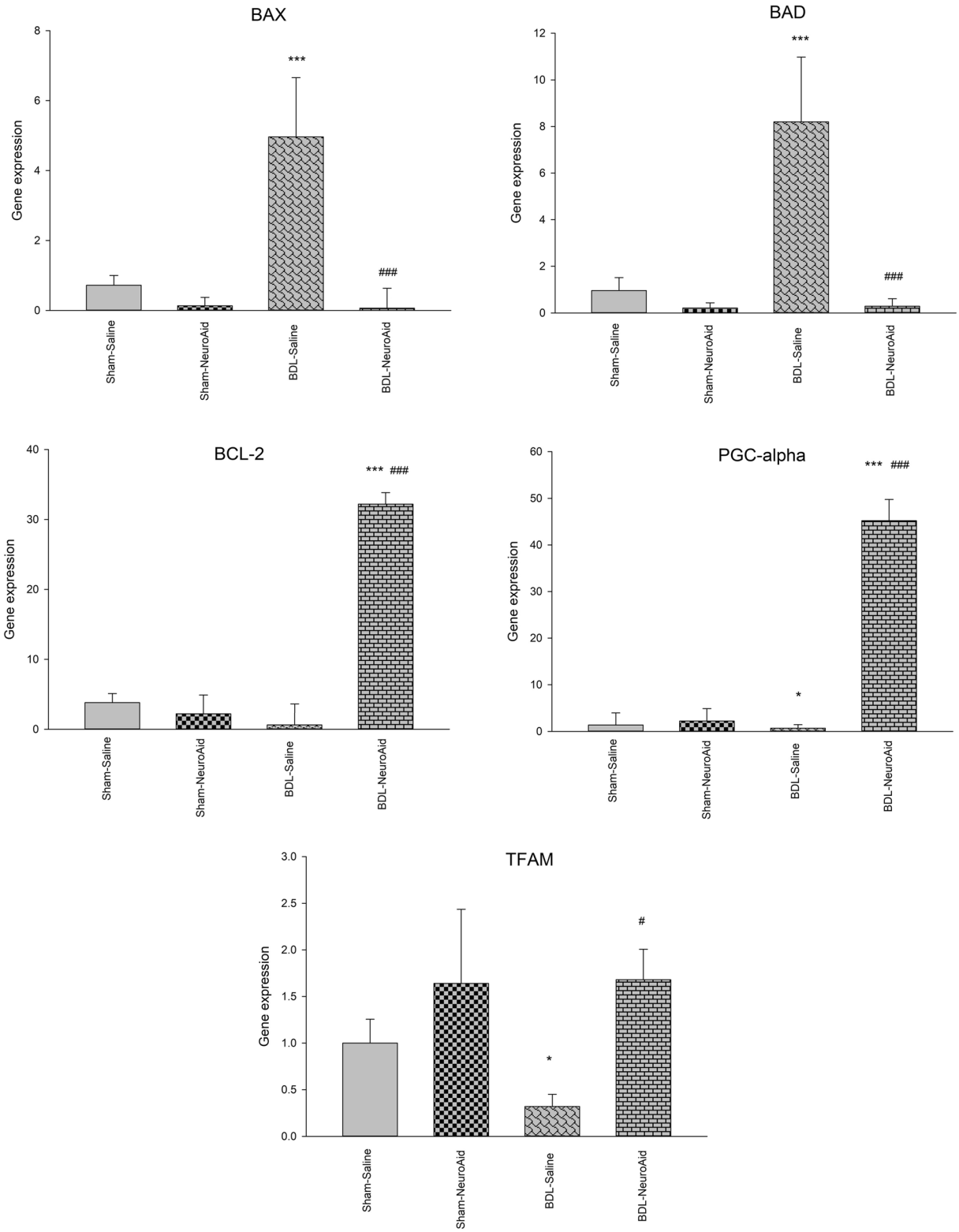

Fig. 3 Shows the results of Real-time PCR analyses for the expression of *BAX*, *BCL-2*, *BAD*, *PGC-1α*, and *TFAM*. Sham-NeuroAid and BDL groups were compared with the sham group. $P < 0.001$*$P < 0.05$ and ***$P < 0.001$ different from the sham-saline group, #$P < 0.05$ and ###$P < 0.001$ different from the BDL-saline group. Two-way ANOVA and post hoc Tukey's were used to analyze data (n = 8)

diseases [18–20]. Previous study has shown that cholestasis-induced glutamatergic disruption impairs memory formation in different brain regions especially the dorsal region of the hippocampus [11]. Previous reports have also suggested that cholestasis changes nitric oxide (NO) level, increases oxidative stress, disrupts calcium homeostasis, and induces cell death; all these mechanisms are involved in memory impairment [70–73]. Additionally, it seems that disruption in the release of corticotrophin-releasing hormone and homeostasis of manganese following cholestasis has a critical role in the induction of cognitive impairments [74–76].

NeuroAid Restored the Impairment Effect of BDL on Spatial Memory

The results showed that NeuroAid restored the impairment effect of BDL on spatial memory (Fig. 2). Previous studies have shown the neuroprotective effects of NeuroAid on memory function. For example, NeuroAid restores fear memory impairment induced by sleep deprivation [77]. NeuroAid also improves cognitive dysfunctions in AD patients [78]. Furthermore, NeuroAid reduces tau phosphorylation [79]. NeuroAid improves performance in novel object recognition, MWM apparatus, and passive avoidance learning task [48]. Interestingly, NeuroAid attenuates hippocampal oxidative stress induced by ROS (reactive oxygen species) accumulation [80]. Also, NeuroAid has neuro-restorative effect that stimulates brain neuro-repair processes including neuroplasticity and neurogenesis [53]. It's important to note that the beneficial effects of NeuroAid may be related to multi-target effects [77]. For example, NeuroAid acts as an activator of K_{ATP} channels [81]. Furthermore, NeuroAid activates the serine/threonine kinase Akt (protein kinase B) pathway in global ischemia model [82]. NeuroAid increases hippocampal neurogenesis via promoting proliferation, neural differentiation, and survival of young neurons [48]. On the other hand, BDNF (brain-derived neurotrophic factor) and VEGF (vascular endothelial growth factor) are critically involved in neuronal repair [83]. BDNF and VEGF are also involved in learning and memory processing [84, 85]. Interestingly, NeuroAid stimulates the expression of BDNF in brain tissue after focal and global ischemia [51]. Furthermore, NeuroAid enhances the expression of VEGF in the hippocampus and cortex of TBI (traumatic brain injury) rats [53]. All these findings show that NeuroAid induces peripheral and central neuroprotective effects in the body, promotes endogenous neural repair processes in the brain, and reverses the impairment effect of BDL on memory.

Increase in BAD and BAX Expression and Decrease in TFAM and PGC-1α Expression Following BDL

The results showed that the expression of *BAD* and *BAX* was increased following BDL (Fig. 3). As mentioned, *BCL-2* family proteins have a crucial role in modulating apoptosis [86]. *BAD* (*BCL-2*-associated death) and *BAX* (*BCL-2*-associated X) are pro-apoptotic proteins that activate caspases via releasing IMS proteins [32]. As the data showed, BDL increased the expression of these genes. BDL accumulates P53 (a gene involved in regulating cell cycle) in the nucleus; while the overexpression of P53 increases the expression of *BAX* and induces apoptosis [87]. Furthermore, maternal obstructive cholestasis during pregnancy (OCP) increases the expression of *BAX* via increase in apoptosis in the OCP placentas [88]. Our previous study has shown that BDL increases *BAX* expression in the striatum of rats [89]. In another study, a dramatic increase in *BAX* expression was observed following BDL [90]. BDL also translocates the cytoplasmic *BAX* to the mitochondria and induces apoptosis via releasing Cytochrome c (Cyt c) into the cytoplasm [91]. Thus, increase in the expression of *BAX* and *BAD* may be related to BDL-induced apoptosis. Additionally, BDL-induced P53 overexpression may lead to the increase in *BAX* expression and apoptosis.

On the other hand, the results showed that the expression of *TFAM* and *PGC-1α* was decreased following BDL (Fig. 3). Previous research has revealed that *TFAM* downregulation following BDL reduces mtDNA copy number in rats [92]. It has been also revealed that long-term cholestasis induces a significant decrease in *TFAM* level [93]. Furthermore, long-term cholestasis decreases *PGC-1α* expression via induction of mitochondrial oxidative stress [94]. In this study, the expression of *TFAM* and *PGC-1α* was decreased following BDL. *TFAM* has a main role in mtDNA maintenance and stabilization [95]. Also, it protects mtDNA from ROS [96]. Note that, activation of *TFAM* is modulated by *PGC-1α* in response to various conditions such as oxidative stress, liver injuries, and BDL [97, 98]. As mentioned before, *PGC-1α* is a master regulator of mitochondrial biogenesis and involved in energy homeostasis [99, 100]. Also, *PGC-1α* has an important role in oxidative stress [101]. We suggest that decrease in *TFAM* level may be related to BDL-induced oxidative stress. *TFAM* is crucial to protect mtDNA from ROS and if the level of ROS gets too high, the function of *TFAM* may be suppressed. Furthermore, the failure of mitochondrial biogenesis following cholestasis may downregulate the expression of *TFAM*. On the other hand, it can be suggested that failure of mitochondrial biogenesis following *TFAM* downregulation [92] reduces *PGC-1α* [102]. Also, decrease of mitochondrial biogenesis and increase of oxidative stress following BDL may suppress the expression of *PGC-1α* [94].

NeuroAid Reversed the Effects of BDL on Genes Expression

Our data showed that NeuroAid reversed all the effects of BDL on the expression of genes (Fig. 3). NeuroAid has neuroprotective properties in rodent models of focal and global ischemia [103]. Previous reports have demonstrated that NeuroAid prevents the death of threatened neuronal tissues [49]. Interestingly, NeuroAid decreases the activity of apoptotic pathways via reducing *BAX* activity in the CA1 pyramidal neurons [49]. NeuroAid also decreases *BAX* expression in the hippocampus [77]. In addition, NeuroAid restores BDL-induced *BAX* upregulation in the striatum of rats [104]. In this research, NeuroAid decreased the expression of pro-apoptotic genes (*BAX* and *BAD*). The limitation of the present study is the lack of immunohistochemical studies to show the exact location of apoptosis within the hippocampus and the exact location of protection. However, we refer to an interesting study. Previous study has reported that NeuroAid decreases the number of injured neurons in the ipsilateral hippocampal CA3 substructure, dentate gyrus, and thalamus following TBI in rats [53]. Furthermore, TBI-induced necrotic and apoptotic neuronal death is significantly reduced after treatment with NeuroAid [53]. GFAP (glial fibrillary acidic protein) is the cell-specific intermediate filament in astrocytes and its upregulation is considered as a definite feature of activated astrocytes [105]. It has been shown that NeuroAid can strongly reduce GFAP expression in both injured cortex and dentate gyrus neurons of TBI rats [53]. Therefore, it seems that NeuroAid induces a neuroprotective effect in both mature neurons and the dentate gyrus (and its subgranular cell layer). Thus, we can suggest that the neuroprotective effect of NeuroAid may also occur in the other important regions of the brain. Additionally, this mentioned study has reported that at 4 h following TBI, the expression of MAP2 (microtubule-associated protein 2) in the CA3 region and dentate gyrus is decreased [53]. MAP2 is expressed in the soma and dendrites of neuronal cells and is critical for microtubule stability and neuroplasticity. Changes in the expression of MAP2 can induce neuronal degeneration following brain injury [106]. Interestingly, NeuroAid prevents MAP2 changes in TBI rats [53]. It has been revealed that NeuroAid can also increase the number of axons of newly generated cells to the CA3 region [53]. We suggest that the decrease in *BAX* and *BAD* expression may be related to the repressive effect of NeuroAid on apoptosis pathways. In addition, NeuroAid via reducing susceptibility of mitochondrial pathways may decrease the expression of *BAX* and *BAD*. On the other hand, the expression of *TFAM* and *PGC-1α* was increased following NeuroAid treatment. NeuroAid by decrease in ROS accumulation attenuates BDL-induced oxidative stress. Thus, we can suggest that NeuroAid increases the expression of *TFAM* via reducing ROS. In addition, it seems that NeuroAid enhances mitochondrial biogenesis. Thus, NeuroAid may increase the expression of *TFAM* via attenuating mitochondrial biogenesis failure. Furthermore, NeuroAid via decrease of oxidative stress and improvement of mitochondrial biogenesis upregulates *PGC-1α*. It can be also suggested that activation of *PGC-1α* plays an important role in the modulation of *TFAM* level.

Conclusions

In conclusion, the results of the present study showed that BDL impaired spatial memory in rats. Furthermore, NeuroAid restored BDL-induced spatial memory impairment. In addition, BDL increased the expression of pro-apoptotic genes (*BAD* and *BAX*) and decreased the expression of *TFAM* and *PGC-1α*. While, NeuroAid reversed all the effects of BDL on genes expression. Note that, our data only shows mRNA and genetic changes but not changes in protein expression. Often, changes in genetic and mRNA levels do not lead to changes in protein levels. Thus, conducting studies using other methods such as western blotting is necessary to complete our conclusions.

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Author Contribution P. Molaei conducted the experiments. S. Vaseghi and M. Hashemi wrote the manuscript and managed the literature search. M. Entezari analyzed data. M. Nasehi designed the study. All authors have approved the final manuscript.

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Data Availability Data will be made available on the reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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